Eesti Maaülikool
Estonian University of Life Sciences

INVASIVE NON-INDIGENOUS CRAYFISH SPECIES AS A THREAT TO THE NOBLE CRAYFISH (*ASTACUS ASTACUS* L.) POPULATIONS IN ESTONIA

INVASIIVSED VÄHI VÕÕRLIIGID JA NENDE OHUSTAV MÕJU JÕEVÄHI (*ASTACUS ASTACUS* L.) ASURKONDADELE EESTIS

KATRIN KALDRE

A Thesis
for application for the degree of Doctor of Philosophy
in Agricultural Sciences

Väitekiri
filosoofiadoktori kraadi taotlemiseks
põllumajandusteaduste erialal

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals. The papers in the thesis are reproduced with the kind permission of the publishers.

I **Kaldre, K.**, Paaver, T., Hurt, M., Grandjean, F. 2017. First records of the non-indigenous signal crayfish (*Pacifastacus leniusculus*) and its threat to noble crayfish (*Astacus astacus*) populations in Estonia. Biological Invasions, 19 (10), 2771–2776.

II **Kaldre, K.**, Meženin, A., Paaver, T., Kawai, T. 2015. A preliminary study on the tolerance of marble crayfish *Procambarus fallax* f. *virginalis* to low temperature in Nordic climate. In: T Kawai, Z Faulkes, G Scholtz, eds. Freshwater Crayfish: A Global Overview, pp. 54-62. CRC Press, Taylor & Francis Group.

III **Kaldre, K.**, Haugjärv, K., Liiva, M., Gross, R. 2015. The effect of two different feeds on growth, carapace colour, maturation and mortality in marbled crayfish (*Procambarus fallax* f. *virginalis*). *Aquaculture International,* 23 (1), 185–194.

IV Gross, R., Kõiv, K., Pukk, L., **Kaldre, K.** 2017. Development and characterization of novel tetranucleotide microsatellite markers in the noble crayfish (*Astacus astacus*) suiTable for highly multiplexing and for detecting hybrids between the noble crayfish and narrow-clawed crayfish (*A. leptodactylus*). *Aquaculture*, 472 (Supplement 1), 50–56.

The contributions from the authors to the papers are as follows:

Paper	Idea	Data collection	Data analysis	Manuscript preparation
I	**KK1,** TP	**KK1,** MH	**KK1,** FG	All
II	**KK1,** TP	AM**, KK1**	**KK1,** AM	**KK1**, TP, TK
III	**KK1,** TP	**KK1,** KH	ML	**KK1**, RG
IV	RG	**KK1,** KK2	KK2, RG, LP	RG

AM: Anton Meženin
FG: Frédéric Grandjean
KH: Kerli Haugjärv
KK1: Katrin Kaldre
KK2: Kuldar Kõiv
LP: Lilian Pukk
MH: Margo Hurt
ML: Mari Liiva
RG: Riho Gross
TP: Tiit Paaver
TK: Tadashi Kawai
All: all authors of the paper

ABBREVIATIONS

bp	Base pair
CPUE	Catch per unit effort (the number of caught crayfish per trap night)
EULS	Estonian University of Life Sciences
HW	Hardy-Weinberg equilibrium
ICS	Indigenous crayfish species
IUCN	International Union for Conservation of Nature
NA	North America
NICS	Non-indigenous crayfish species
PEAR	Paired-end read merger
PCR	Polymerase chain reaction
PFU	PCR forming units
qPCR	Quantitative (real-time) polymerase chain reaction
SSR	Simple sequence repeat
STR	Short tandem repeats

1. INTRODUCTION

The noble crayfish (*Astacus astacus*) is from the point of view of recreational fishing and consumption the most highly valued freshwater crayfish species in northern Europe. Once abundant, it has suffered from a long-term population decline due to introduced alien crayfish species, crayfish plague, habitat loss and over-harvesting. It is now considered threatened according to the Bern convention, the IUCN Red List and protected in EU legislation by Council Directive No. 92/43/EEC (Edsman *et al*. 2010; Kozák *et al*. 2015). In Estonia it is the only indigenous decapod crayfish species. In the wild, the noble crayfish occurs in Estonia in more than 255 lakes and stretches of river, but most sites have low densities, except for some populations in South-Eastern Estonia and on the island of Saaremaa (Paaver & Hurt 2009). Thus, it is in need of protection from alien species.

The introduction of non-indigenous crayfish species (NICS) is one of the major causes of extinction of all indigenous crayfish species (ICS) in European freshwaters (Holdich *et al*. 2009). NICS of North American (NA) origin are more aggressive, more fertile and more tolerant to environmental changes than native crayfish species (Souty-Grosset *et al.* 2006). However, their most devastating effect on ICS stocks is due to the fact that they are latent carriers of the crayfish plague (Alderman *et al*. 1990), which is caused by the oomycete *Aphanomyces astaci* (Unestam 1972). In most cases, crayfish plague is lethal to all crayfish species not originating from NA, including the indigenous noble crayfish (OIE 2012). Crayfish plague spread to Estonia in 1896 (Järvekülg 1958) and it has destroyed many noble crayfish populations since then. However, until 2006, crayfish plague outbreaks were recorded only on mainland Estonia, they were never detected on the island of Saaremaa, which was a unique place in the whole of Europe, with abundant noble crayfish populations and no alien crayfish species (Paaver & Hurt 2009). The situation has now changed and both the crayfish plague and the NA signal crayfish (*Pacifastacus leniusculus*) have been found on the island of Saaremaa. As there can be other causes of mass mortality of crayfish, it is important to confirm the crayfish plague outbreaks using appropriate methods. Modern molecular methods and genetic markers provide useful tools for the detection and quantification of the causative agent of crayfish plague *Aphanomyces astaci* (Vrålstad *et al.* 2009) and for identification of its strains (Grandjean *et al.* 2014).

The signal crayfish is the most widespread NICS in Europe (Kouba *et al*. 2014). Until 2008 Estonia was one of the last countries, together with Ireland, Norway, Croatia, Slovakia and Russia, where alien crayfish species were not recorded (Kouba *et al*. 2014). Currently, signal crayfish are already present in Estonia and also in all other European countries (except Ireland), where freshwater crayfish species occur. Due to its potential devastating impact on the indigenous noble crayfish populations, it is very important to study the spread and the status of signal crayfish populations in Estonia.

Another threat to ICS may be the trade in live ornamental freshwater crayfish. This has grown rapidly in the last decade, and has become the major pathway for new NICS introductions into Europe (Chucholl *et al*. 2012). One of these is the marbled crayfish (*Procambarus fallax* f. *virginalis*) (Scholtz *et al*. 2003; Martin *et al*. 2010) that was discovered for the first time in the German and Austrian aquarium trade in the mid-1990s (Lukhaup 2001), and is now also available in the Estonian aquarium trade. Currently, it has spread into the natural environment and is rapidly spreading in Central European countries due to its parthenogenetic reproduction method, creating a new threat for European indigenous water ecosystems (Souty-Grosset *et al*. 2006; Chucholl & Daudey 2008; Chucholl 2011). To assess the potential threat of the marbled crayfish to Estonian water ecosystems, it is important to evaluate its tolerance to low water temperatures during winter and other biological traits such as growth and maturation.

Based on the dangers of NICS, marbled crayfish together with signal crayfish, red swamp crayfish, spiny-cheek crayfish and virile crayfish are now included on the list of invasive alien species of Union concern pursuant to Regulation (EU) No 1143/2014 of the European Parliament and of the Council. They cannot legally be imported, kept, bred, transported, sold, used or exchanged, allowed to reproduce, grown or be cultivated, or released into the environment.

The Estonian Nature Conservation Act is intended to protect the indigenous species of Estonia and to prevent the introduction and spread of non-native species in the wild. It is permitted to cultivate only the noble crayfish in Estonia. The law specifically prohibits the introduction of signal crayfish, narrow-clawed crayfish (*Astacus leptodactylus*) and spiny cheek crayfish (*Orconectes limosus*), which are widespread in the water

bodies of the neighbouring countries to Estonia (Kouba *et al*. 2014). Unfortunately, two of these species are already present in Estonia: the first signal crayfish and spiny cheek crayfish were detected in 2008 and in 2017 respectively. However, the potentially dangerous marbled crayfish is not yet listed in the Estonian Nature Conservation Act as a prohibited in Estonia species.

Knowledge of the intra-specific genetic diversity and the genetic background of populations when selecting material for re-introduction, supplemental stocking or captive breeding is crucial for the protection of the genetic resources of indigenous species. To obtain this information, appropriate molecular tools (genetic markers) are required. Similarly, genetic markers are required for reliable discrimination of morphologically similar species and their hybrids. The first microsatellite markers for *A. astacus* (a total of 19 dinucleotide repeat loci) were developed by Kõiv *et al*. (2008, 2009) and have been applied subsequently in several studies of genetic diversity (Gross *et al*. 2013; Schrimpf *et al*. 2014; Blaha *et al*. 2016). However, the number of practically usable loci in these studies was rather low (from six to 10 loci), because many loci failed to amplify consistently, or exhibited extensive stuttering that made their reliable genotyping difficult. For *A. leptodactylus*, there were no published microsatellite loci available. Therefore, it is important to develop a new set of polymorphic microsatellite markers for *A. astacus* that at least partially cross-amplify also in *A. leptodactylus,* and possess species-specific allele size ranges for the identification of potential hybrids between these sister species.

This doctoral dissertation is based on four papers which consider the threat of NICS to noble crayfish in Estonia. In the first paper (**I**), I give an overview of the status and distribution of signal crayfish illegally introduced to Estonia and their possible role in a series of crayfish plague outbreaks which have recently taken place in the noble crayfish populations in Estonia. In the second paper (**II**), I discuss the tolerance of marbled crayfish to low temperatures as a factor potentially limiting the spread of that species in North-European countries. In the third paper (**III**) the effect of two types of feed with different contents of dietary astaxanthin on carapace colouration, growth rate, maturation and mortality in marbled crayfish was estimated. These parameters may influence the potential success of colonization of North-European waters by this species. In the fourth paper (**IV**), a set of polymorphic tetranucleotide repeat microsatel-

lite markers was developed for noble crayfish and narrow-clawed crayfish that can be used for the reliable identification of these species, and their hybrids, along with estimation of the genetic diversity and differentiation of their populations. This allows for the better protection of the genetic resources of the indigenous noble crayfish in Estonia.

2. REVIEW OF LITERATURE

2.1. Consequences of the spread of NICS in Europe

Crayfish have played an important role in social and cultural activities in Europe since the Middle Ages when it was important in the diet of both common people and aristocrats (Souty-Grosset *et al*. 2006). Crayfish consumption was common in Europe, people captured and cooked them and trade with crayfish was widespread until the end of the 19th century (Pöckl 1999; Souty-Grosset *et al*. 2006). Even today noble crayfish and narrow-clawed crayfish are used for human consumption in Europe (Harlioğlu 2011). In addition, crayfish and their body parts (particularly the gastroliths) have been used in popular medicine for centuries (Swahn 2004). Crayfish are dominant decapods in many freshwater habitats because of their large size, mobility, behaviour and omnivory; they are bioindicators and indicators for water quality (Reynolds *et al*. 2013).

There are about 650 freshwater crayfish species in the world. Five indigenous astacid species – noble crayfish, narrow-clawed crayfish, thick-clawed crayfish *Astacus pachypus*, white-clawed crayfish *Austrapotamobius pallipes* and stone crayfish *Austrapotamobius torrentium* live in Europe (Souty-Grosset *et al*. 2006). These species still inhabit Europe despite drastic reductions in their populations since the second half of the 19th century. This is due to physical habitat modification, climate change effects such as droughts, land-use activities, toxic substances, overfishing, but mainly the spread of crayfish plague by introduced invasive NICS (Bohman *et al*. 2006; Allan & Castillo 2007; Koutrakis *et al*. 2007; Kozubíková *et al*. 2008; Füreder 2015).

In some areas, the ICS have suffered greatly from the introduction of NICS and are listed in the IUCN Red List of Threatened Species as endangered or vulnerable species (Füreder 2015). These species are the white-clawed crayfish in France, Italy, Spain, Britain and Ireland, the stone crayfish in Austria, Germany, Croatia and Switzerland, and the noble crayfish in Austria, Germany and Switzerland (Füreder 2015). The noble crayfish is also listed as vulnerable in the IUCN Red List of Estonia, but it is not protected by the Estonian Nature Conservation Act (Paaver & Hurt 2009). This means, that it can be caught for consumption on a limited scale.

The spreading of species (termed as 'none-native', 'non-indigenous', 'alien' or 'invasive alien species' in different studies) outside their native area, which has been mediated by humans causes changes in the ecosystems and has negative impacts on the biodiversity of systems to which they are introduced (Jeschke *et al*. 2014). These changes can be dramatic and may result in the extinction of native species or radical changes in ecosystem functioning (Simberloff *et al*. 2013; Jeschke *et al*. 2014).

The main reason for the introduction of NICS in Europe was their expected commercial use in aquaculture and harvest for human consumption (Holdich *et al*. 2009; Kotovska 2016). Three NICS of NA origin – spiny-cheek crayfish, signal crayfish and red swamp crayfish *Procambarus clarkii* were introduced into Europe between 1890 and the mid-1970s, and became widespread across the continent (Füreder 2015).

Two the most prevalent NICS in northern Europe are the signal crayfish and the spiny-cheek crayfish (Souty-Grosset *et al*. 2006), both of which are widespread in countries neighbouring Estonia (Souty-Grosset *et al*. 2006; Holdich *et al*. 2009; Kouba *et al*. 2014). The signal crayfish has invaded 29 European countries, and the spiny-cheek crayfish has been recorded in at least 22 (Kouba *et al*. 2014). Estonia, together with Slovakia, Norway, Croatia and Russia are newly confirmed countries with signal crayfish (Füreder 2015). The red swamp crayfish has invaded 15 European countries, and is widespread in Southern Europe, particularly Portugal and Spain (Holdich *et al*. 2009, Oscoz *et al*. 2010).

Currently, the introductions of new NICS (introduced after 1980's) that are usually kept as ornamental animals in aquaria is taking place (Holdich *et al*. 2009; Kouba *et al*. 2014). Most of the traded species originate from NA, and may either accidentally escape or are intentionally released into the wild by their keepers (Chucholl 2013; Kotovska 2016). This has already been reported in several countries in the world (Feria & Faulkes 2011; Chucholl 2013; Faulkes 2015). Six NA species of the family *Cambaridae* (genera *Orconectes* and *Procambarus*) have been recorded from European freshwaters: calico crayfish *Orconectes immunis*, Kentucky River crayfish *Orconectes juvenilis,* virile crayfish *Orconectes* cf. *virilis*, white river crayfish *Procambarus* cf. *acutus*, marbled crayfish *Procambarus fallax* f. *virginalis* and Florida crayfish *Procambarus alleni*. Two Australian crayfish species of the genus *Cherax* (*Parastacidae*) – the yabby *Cherax destructor* and redclaw *Cherax quadricarinatus* were brought to

Europe due to aquaculture interests (Füreder 2015). These new NICS have established populations in the wild, making them a new threat to the local ecosystems (Kawai *et al*. 2009; Feria & Faulkes 2011, Füreder 2015).

Due to the parthenogenetic reproduction, the marbled crayfish is potentially the most invasive of all marketed species (Scholtz *et al*. 2003; Patoka 2014).

2.2. The studied species

2.2.1. The noble crayfish *Astacus astacus*

The noble crayfish became widespread after the last ice age (Souty-Grosset *et al*. 2006) and occurs in 39 territories of the European continent today (Kozák *et al*. 2015) (Fig. 1). It inhabits a wide variety of habitats including lakes, ponds, reservoirs, streams and rivers, in both lowlands

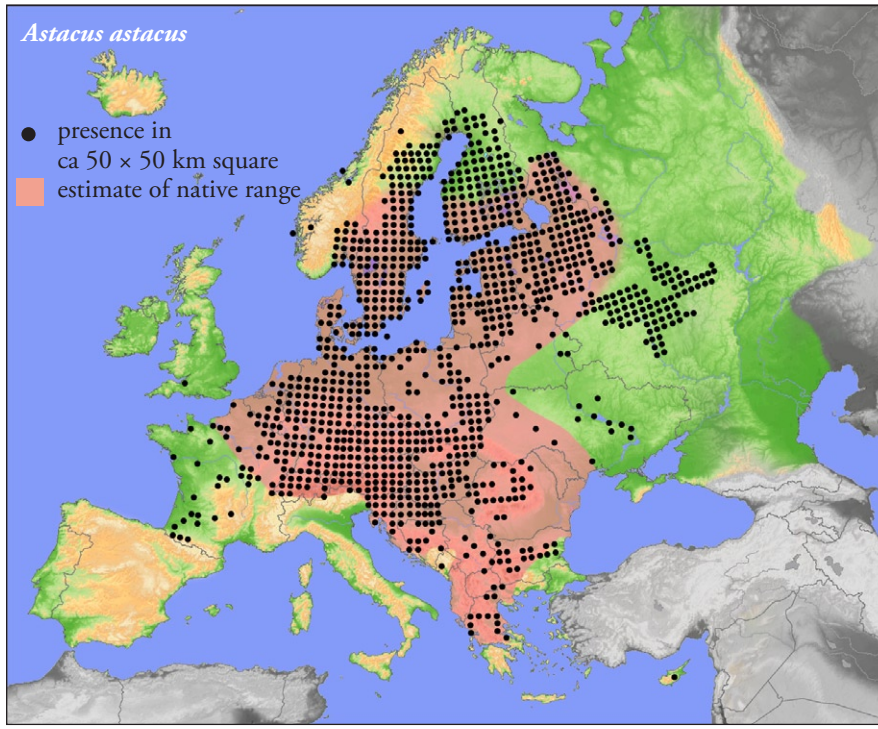

Figure 1. Noble crayfish distribution in Europe. Orange background represent original distribution of noble crayfish (Kouba *et al.* 2014).

and hills (Füreder 2015). This species prefers localities with stones and roots, where shelter availability is high, and dig burrows in clay and earth banks (Souty-Grosset *et al*. 2006). They avoid muddy bottoms as their habitat, but they use such places for foraging (Skurdal & Taugbųl 2002; Holdich *et al*. 2006).

The noble crayfish is sensitive to both pollution and physical damage of the environment (Souty-Grosset *et al.* 2006). In localities where both noble crayfish and signal crayfish can be found together, noble crayfish will eventually be displaced or succumb to crayfish plague (Souty-Grosset *et al.* 2006).

The optimal growth temperature of the noble crayfish is 16–24 °C, while 28 °C can be tolerated for a short time period (Füreder 2015). The noble crayfish does not tolerate oxygen deficiency. Oxygen concentrations between 3–4 mg/l^{-1} are minimum values (Füreder 2015).

The noble crayfish may live for more than 20 years and grow to a size up to 18 cm, but this is very rare. Usually specimens below 15 cm total length are caught (Souty-Grosset *et al*. 2006).

Females reach sexual maturity at size ranges from 5.8 to 6.2 cm total length at an age of 4 years (Souty-Grosset *et al.* 2006). Males become sexually mature in the third year of their life, at a total body length of around 7.0 cm (Souty-Grosset *et al.* 2006). The sexually mature males moult once or twice per year and the females only once (Souty-Grosset *et al.* 2006). Under the conditions of the Scandinavian countries, incubation lasts from 8 to 9 months (Kozák *et al.* 2015). The average number of eggs is between 90 and 260, of which about 55% to 90% hatch; hatching depends to a great extent on the size and age of the female (Souty-Grosset *et al.* 2006).

The colour of noble crayfish is mostly brownish red, but can also be greenish, blackish or bluish (Souty-Grosset *et al.* 2006). Claws are big and strong, especially in males. The carapace is smooth but, differently from the signal crayfish, there is a sharp stud on both sides at the joint between the head and thorax (Souty-Grosset *et al*. 2006).

2.2.2. The signal crayfish *Pacifastacus leniusculus*

The NA signal crayfish is the most widespread NICS in Europe, having been first introduced into Sweden in 1959 (Fig. 2) (Kouba *et al.* 2014).

In Europe signal crayfish inhabit similar environments to ICS, particularly noble crayfish (Souty-Grosset *et al.* 2006). The signal crayfish is more aggressive than ICS (Souty-Grosset *et al.* 2006). *P. leniusculus* appears to out-compete *A. astacus* for shelter (Souty-Grosset *et al.* 2006), it usually has a wider ecological amplitude, and withstands more extreme conditions (Souty-Grosset *et al.* 2006). It is also able to survive in warm water localities, where the temperature reaches 30 °C, but they do not prosper in very cold localities such as in northern Scandinavia (Kozák *et al*. 2015). The signal crayfish are more tolerant to pollution and brackish water (Souty-Grosset *et al.* 2006; Kozák *et al*. 2015). They are highly resistant to crayfish plague, for which it acts as a carrier (Souty-Grosset *et al*. 2006).

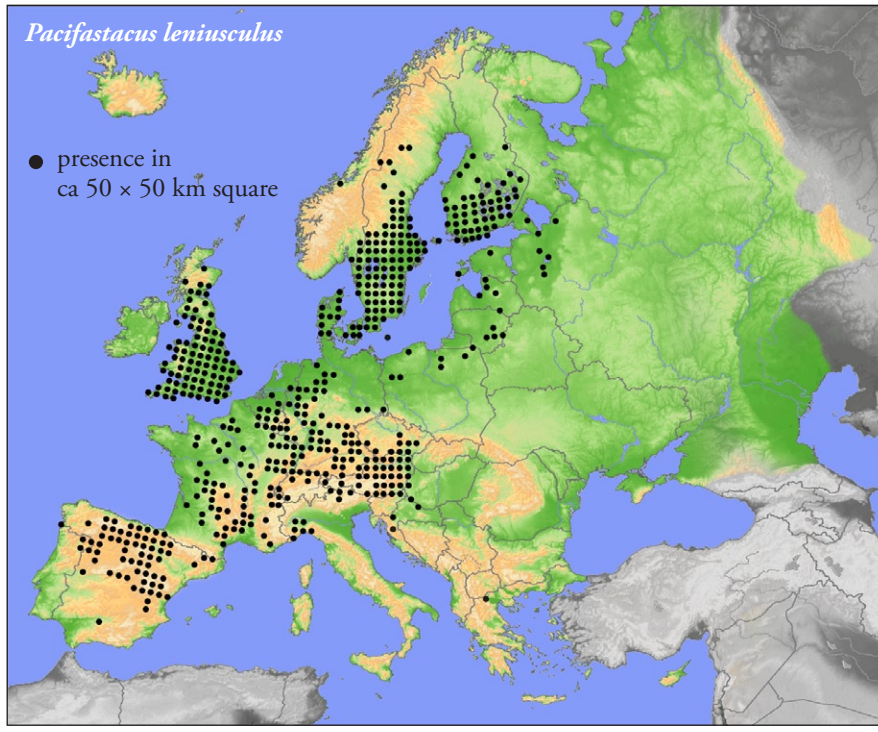

Figure 2. Signal crayfish distribution in Europe (Kouba *et al.* 2014).

The signal crayfish can live more than 20 years and grow up to 16 cm long (Souty-Grosset *et al*. 2006; Kozák *et al.* 2015). Females reach sexual maturity in their third year and males in the second year at sizes ranging from 6 to 9 cm (Souty-Grosset *et al.* 2006). The average number of eggs range from 200 to 400 (Souty-Grosset *et al.* 2006). The signal crayfish juveniles hatch 3 to 4 weeks earlier than noble crayfish under the same conditions (Kozák *et al.* 2015).

The colour of signal crayfish varies according to habitat, but is usually bluish-black to reddish-brown (Souty-Grosset *et al.* 2006). Claws are shorter and wider, thicker and smoother than those of noble crayfish, and red on the underside (Souty-Grosset *et al.* 2006). A light blue to white patch can be found at the joint of the fingers of the claw – the so-called signal that gave the species its name (Souty-Grosset *et al*. 2006).

2.2.3. The marbled crayfish *Procambarus fallax* f. *virginalis*

Until 2017 it was considered that marbled crayfish is a special form, closely related to *Procambarus fallax,* and its natural habitat is unknown, although *P. fallax* is a subtropical American species (Martin *et al.* 2010). Recent genetic analyses have revealed that a marbled crayfish genomic pile-up happened during their rearing in aquaria, and it should be considered an independent species named scientifically *Procambarus virginalis* (Lyko 2017; Gutekunst *et al*. 2018).

During the last decade, it has become a very popular species among hobby aquarists and researchers (Vogt 2008, 2010; Faulkes 2010) due to their appealing colouration, undemanding variety of environmental conditions and reproduction (Chucholl *et al*. 2012).

The marbled crayfish is a triploid organism and reproduces parthenogenetically (Martin *et al.* 2015). The populations consist of only females, which are genetically identical (Martin *et al.* 2015). Thus, it could be an excellent test object for feeding, temperature tolerance and colouration studies as all specimens are genetically identical. However, they show surprisingly broad ranges in variation of colouration, growth, lifespan, reproduction, morphometric parameters, behaviour and fluctuating asymmetry of sense organs, even when reared under identical conditions (Martin *et al.* 2007; Vogt 2008).

Marbled crayfish can grow up to a size of 13 cm, but most often less than 10 cm (Souty-Grosset *et al.* 2006). They are best cultured at temperatures of 18–25 °C, but can withstand temperatures below 8 °C and above 30 °C for many weeks, although reproduction stops and mortality increases under these conditions (Vogt *et al.* 2004; Kaldre *et al.* 2016). The first spawning of marbled crayfish occurs on average at an age of 25 weeks when reared at 25 °C, and after 35 weeks at 20 °C (Vogt *et al.* 2004). Maturity is reached at size around 40 mm of total body length (Vogt *et al.* 2004). In suiTable rearing conditions they can reproduce throughout the entire year at intervals of only 8 to 9 weeks, and their incubation period is only 2 to 3 weeks (Holdich *et al.* 2006; Vogt 2010). Each marbled crayfish can produce between 30 and 400 juveniles per batch, depending on their age and size (Vogt 2008). In laboratory conditions they can live up to 3 years (Pöckl *et al.* 2006; Vogt 2010).

Due to human activities, marbled crayfish have spread to several countries in the world and have successfully established populations in the

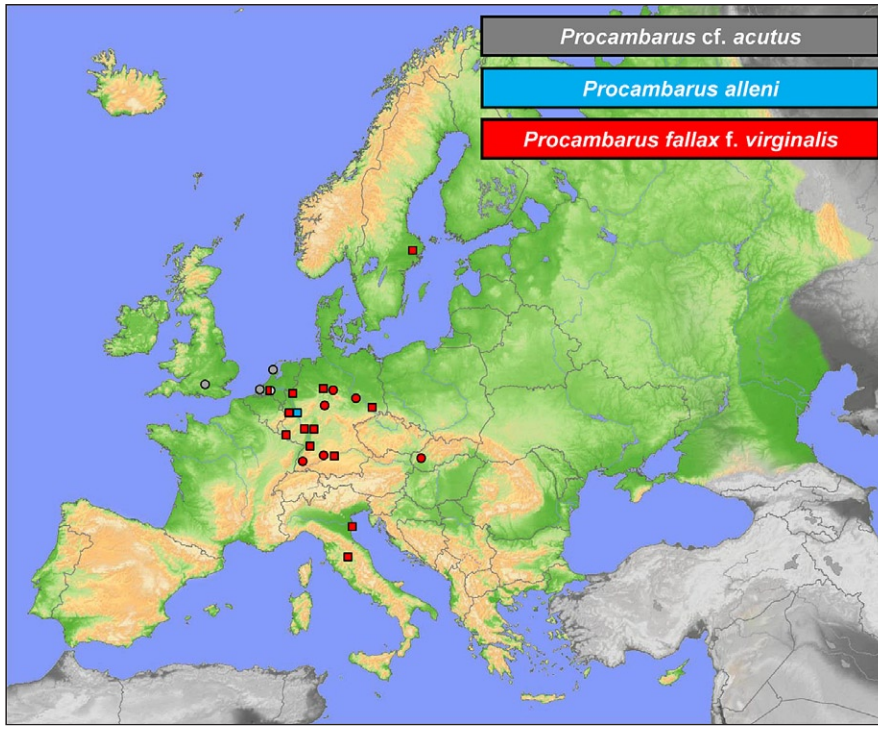

Figure 3. Distribution of marbled crayfish and other cambarid species in Europe (Kouba *et al.* 2014).

wild (Jones *et al*. 2009; Kawai *et al*. 2009; Kawai & Takahata 2010; Feria & Faulkes 2011). Karplus *et al*. (1998) suggest that temperate and sub-tropical crayfish are not resistant to low temperatures, but finding marbled crayfish in natural temperate waters indicates that they tolerate a wide range of environmental conditions.

In the wild, marbled crayfish were first recorded in Germany in 2003 (Marten *et al*. 2004). Since then, their presence has been reported from various European countries, including the Netherlands, Italy, Slovakia, Croatia, and even Sweden (Chucholl *et al*. 2012; Kouba *et al*. 2014; Samardžić 2014) (Fig. 3), and they are apparently well established in Madagascar (Jones *et al.* 2009; Kawai *et al.* 2009). The marbled crayfish are also carriers of crayfish plague (Keller *et al*. 2014; Mrugała *et al*. 2015).

In Estonia marbled crayfish is a popular aquarium species. To date, fortunately, marbled crayfish have not been found in the wild in Estonia.

2.2.4. The narrow-clawed crayfish *Astacus leptodactylus*

The narrow-clawed crayfish is indigenous to Europe and has a wide habitat preference (Fig. 4). It is known in 32 European territories, and the current distribution of *A. leptodactylus* and *A. astacus* overlaps to a great extent, though the main centre of their occurrence is in Central and Northern Europe, and in Eastern Europe and the Middle East, respectively (Kouba *et al.* 2014). However, it is missing from Estonia and can be treated here as an alien species. It is more active in daylight, more tolerant to environmental conditions, less sensitive to pollution and their temperature tolerance is wider than in other European ICS (Füreder 2015).

The narrow-clawed crayfish inhabit both brackish and freshwaters (Souty-Grosset *et al.* 2006). They can live for more than 10 years and are a relatively fast growing species (Souty-Grosset *et al*. 2006). Males can grow up to 30 cm in total length, but usually they grow up to 15 cm (Kozák *et al*. 2015). They reach sexual maturity in their third or fourth year of life at a total body length from 7.5–8.5 cm (Souty-Grosset *et al.* 2006). They have high fecundity, 200–800 eggs per female (Souty-Grosset *et al*. 2006).

The colour of narrow-clawed crayfish varies a great deal according to habitat, but honey brown or olive green specimens are the most common

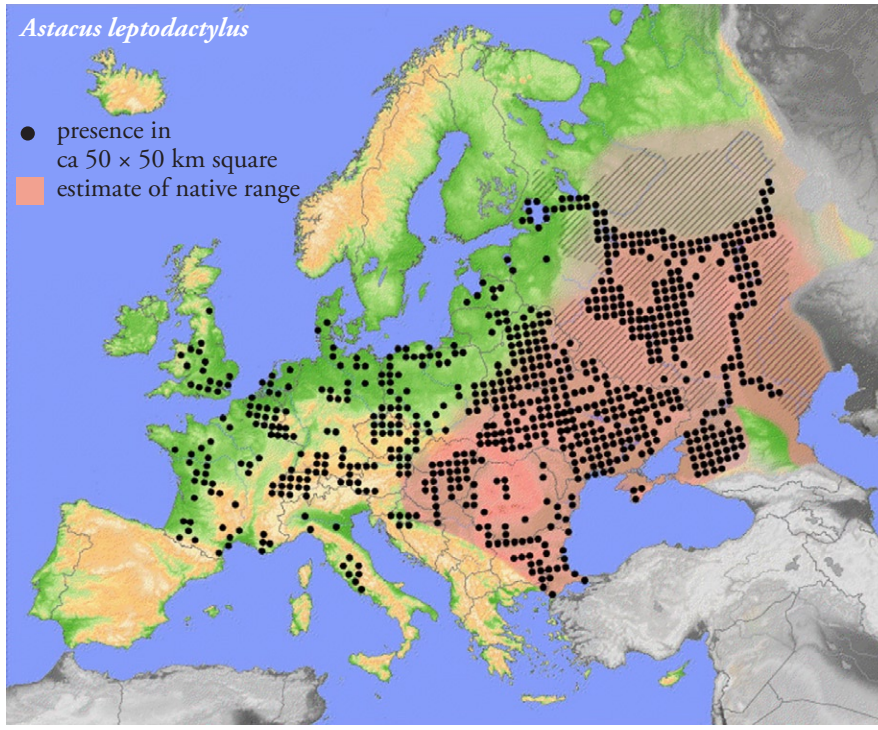

Figure 4. Narrow-clawed crayfish distribution in Europe. Orange background represent original distribution of narrow-clawed crayfish (Kouba *et al.* 2014).

(Souty-Grosset *et al.* 2006). The shell is coarse. Claws are scissor-like in appearance, long, narrow and straight (Souty-Grosset *et al.* 2006).

2.3. Crayfish plague

2.3.1. Causative agent, history, life cycle and symptoms

Crayfish plague (*Aphanomyces astaci*) is the most destructive disease of European crayfish, because it is highly infectious and can devastate the whole crayfish population (Cerenius *et al*. 2009). Crayfish mass mortalities were first reported in Europe in 1859 in Italy, and spread to other European countries over the following three decades (Alderman 1996). Crayfish plague reached Estonia in 1896 (Järvekülg 1958). The source of the introduction of crayfish plague into Europe is unknown, but it has been speculated that it could have been the North-American spiny-

cheek crayfish which was introduced to Europe in 1890 (Souty-Grosset *et al*. 2006) or transferred accidentally in the ballast water tank of a ship crossing the Atlantic (Alderman 1996).

Crayfish plague is caused by an oomycete *Aphanomyces astaci* (Unestam 1972; Söderhall & Cerenius 1999) that exists in three forms – mycelium (in the crayfish cuticle), a cyst, and a swimming zoospore (in the ambient water) (Makkonen 2013). The infective stage is the zoospore, which is capable of locating the host, attaching and encysting on its cuticle and initiating infection (Souty-Grosset *et al*. 2006). The zoospores primarily infect crayfish, although there are some recent studies that have shown that freshwater crabs and shrimps may be carriers of *A. astaci* (Schrimpf *et al*. 2014; Svoboda *et al*. 2014).

A. astaci is not adapted to living outside hosts, and zoospores are short lived (Unestam 1966). They survive only for several days or a week depending on the temperature (Unestam 1966). The zoospores need wet conditions, due to *A. astaci* being extremely sensitive to drying out (Alderman & Polglase 1986). Therefore, contaminated water, items that have been in contact with contaminated water, and also animals that have been feeding on the infected crayfish could be one of the pathways of transmission of *A. astaci* (Oidtmann *et al*. 2002).

Most of the latent carriers of the crayfish plague are NA origin NICS, being generally more resistant to infection themselves (Alderman *et al*. 1990; Persson & Söderhäll 1983), because of their stronger immune systems that inhibit the parasite's growth in the cuticle and prevent its growth in the body (Cerenius *et al*. 2003). The parasite remains alive, reproduces in the crayfish and may transmit to other hosts (Söderhall & Cerenius 1999), thus NICS from NA represent chronic carriers of the crayfish plague (Kozák *et al.* 2015).

Crayfish plague is lethal in most cases to all freshwater crayfish species not originating from the NA (Alderman & Polglase 1988).

Infected ICS have disoriented movements, attempt to scratch themselves, have an impaired escape reflex, show loss of co-ordination and fall onto their backs being unable to right themselves (Alderman *et al.* 1987). The first sign of an outbreak may be the presence of large numbers of dead crayfish in a river or lake (Alderman *et al.* 1987).

2.3.2. Molecular tools for detection of crayfish plague

The former crayfish plague diagnostics was based on the microbiological isolation of *A. astaci* from diseased crayfish and the demonstration of its virulence through re-infection attempts (Alderman & Polglase 1986; Cerenius *et al*. 1988), but it is time consuming, complicated and often an unsuccessful process.

The development of polymerase chain reaction (PCR) based molecular methods has facilitated reliable detection of crayfish plague. It enables the detection of *A. astaci* directly in the samples of the crayfish cuticle and is applicable both for crayfish dying of crayfish plague and also for carriers of the parasite with no symptoms of the disease (Oidtmann *et al*. 2006; Vrålstad *et al*. 2009). At present, the World Organization for Animal Health recommends using the quantitative PCR (qPCR) method of Vrålstad *et al*. (2009) as the most reliable. The TaqMan® probe targets the internal transcribed spacer (ITS) regions of the nuclear ribosomal gene cluster unique to *A. astaci* (Vrålstad *et al.* 2009). This method not only provides information on the presence of the pathogen DNA, but additionally allows quantification of the target DNA, and thus estimation of the agent level in crayfish (Vrålstad *et al.* 2009). This method is highly sensitive and specific (Vrålstad *et al.* 2009). The cross reactions or false positives with the closely related *Aphanomyces* species are exluded (Vrålstad *et al*. 2009; Tuffs & Oidtmann 2011), but this method does not allow for the investigation of the genetic diversity of *A. astaci*.

A. astaci produces genetically, physiologically and ecologically different genotype groups of the pathogen, and their occurrence is related to the different crayfish species. Five main genotype groups have been discovered to date (Table 1) (Grandjean *et al*. 2014).

Recent studies have indicated that genotype group A causes lower mortality rates in susceptible noble crayfish than the other genotype groups of which the hosts are NICS (Makkonen *et al.* 2012, 2014; Viljamaa-Dirks *et al.* 2011, 2013; Becking *et al*. 2015). This might be the result of the genetic adaptation of noble crayfish to the A-genotype over its more than 100-year co-existance (Makkonen *et al*. 2012).

Grandjean *et al*. (2014) developed nine microsatellite markers for *A. astaci* (Aast 2, 4, 6, 7, 9, 10, 12, 13, 14) that separate all five known genotypes

Table 1. Genotypes of *A. astaci* (Makkonen 2013)

Genotype	Original host	Reference
A	unknown	Huang *et al*. (1994)
B	*P. leniusculus* (Lake Tahoe)	Huang *et al*. (1994)
C	*P. leniusculus* (Lake Pitt)	Huang *et al*. (1994)
D	*P. clarkii*	Diéguez-Uribeondo *et al*. (1995)
E	*O. limosus*	Kozubíková *et al*. (2011a)

of *A. astaci* (defined previously using random amplified DNA (RAPD) methodology) and can even reveal possible subgroups within the genotype groups (Maguire *et al*. 2016). This method can be applied to cuticle samples, but successful PCR amplification assumes at least the A3 level of *A. astaci* DNA, revealed using the qPCR method (Grandjean *et al*. 2014).

2.4. Microsatellite markers

Microsatellites are a very popular type of genetic marker in population genetic studies due to their high degree of polymorphism, random distribution across genomes and neutrality with respect to selection (Avise 1994; Jarne & Lagoda 1996; Bohonak 1999; Liu & Cordes 2004; Selkoe & Toonen 2006; Chauhan & Rajiv 2010). They are short tandem repeats (STR) of 1–6 nucleotides in DNA sequences that can vary in length between 5 and 40 repeats, but longer strings of repeats are also possible. In molecular genetic studies, di-, tri- and tetranucleotide tandem repeats are the most common. Microsatellites exhibit a high mutation rate of 10^{-2} to 10^{-6} nucleotides per locus per generation, which generates the high levels of allelic diversity necessary for genetic studies of processes acting on ecological time scales (Schlötterer 2000).

The first microsatellite markers for *A. astacus* (a total of 19 dinucleotide repeat loci) were developed by Kõiv *et al*. (2008, 2009) and have been applied subsequently in several studies of genetic diversity (Gross *et al*. 2013; Schrimpf *et al*. 2014; Blaha *et al*. 2016). However, the number of practically usable loci in these studies was rather low (from six to 10 loci), because many loci failed to amplify consistently, or exhibited extensive stuttering that made their reliable genotyping difficult. Microsatellite

loci have been developed also for the signal crayfish (Azuma *et al.* 2011; Froufe *et al.* 2015) and the marbled crayfish (Vogt *et al.* 2015) but not yet for the narrow-clawed crayfish.

The main challenge in the past was the high cost and large effort of developing microsatellite markers for each species using traditional molecular genetic methods (Ostrander *et al.* 1992). However, this has now been alleviated with the advent of next generation sequencing, which allows the detection and characterization of large numbers of microsatellite loci in any species much more efficiently (Guichoux *et al.* 2011).

3. HYPOTHESES AND AIMS OF THE STUDY

The main objective of this thesis was to give an overview of invasive non-indigenous crayfish species in Estonia and to assess their potential threats to the indigenous noble crayfish populations, including the problem of the spreading of crayfish plague.

The specific aims of the thesis are:

1) to describe the status and distribution of alien invasive signal crayfish in Estonia and their role in a series of plague outbreaks which have recently taken place in indigenous noble crayfish populations (**I**);

   Hypothesis: *Recent crayfish plague outbreaks in the noble crayfish populations in Estonia have at least partially been related to the spreading of NICS*.

2) to estimate the effect of low temperature and different feeds on survival, growth, reproduction, behaviour and carapace colouration of marbled crayfish and to assess its threat to Estonian noble crayfish populations (**II, III**);

   Hypothesis: *Marbled crayfish is potentially dangerous to the noble crayfish populations in Estonia because it grows and reproduces well in different conditions, and can tolerate low water temperatures during winter*.

3) to develop a new set of polymorphic tetranucleotide repeat microsatellite markers for indigenous *Astacus astacus,* and potentially invasive *Astacus leptodactylus,* for reliable identification of potential hybrids between these sister species **(IV)**;

   Hypothesis: *Using next generation sequencing technologies it is possible to develop a set of polymorphic microsatellite markers for A. astacus that at least partially cross-amplify also in A. leptodactylus and possess species-specific allele size ranges that allow identification of potential hybrids between these sister species.*

4. MATERIAL AND METHODS

4.1. Methods of detection of signal crayfish in Estonia (I)

The first three signal crayfish populations in Estonia were found during annual monitoring of the status of noble crayfish populations carried out by the Chair of Aquaculture of the Estonian University of Life Sciences (EULS). The fourth signal crayfish population was found by local fishermen. After the initial discovery of signal crayfish, monitoring was conducted at all these sites yearly (Fig. 5; Table 2). The test fishings were usually carried out with cylindrical Mjärde Lini traps that were applied as lines and kept in the water overnight. Frozen fish was used as bait. For every trapping session CPUE was recorded at each site (Table 2). Information about the occurrence of noble crayfish in these rivers before the

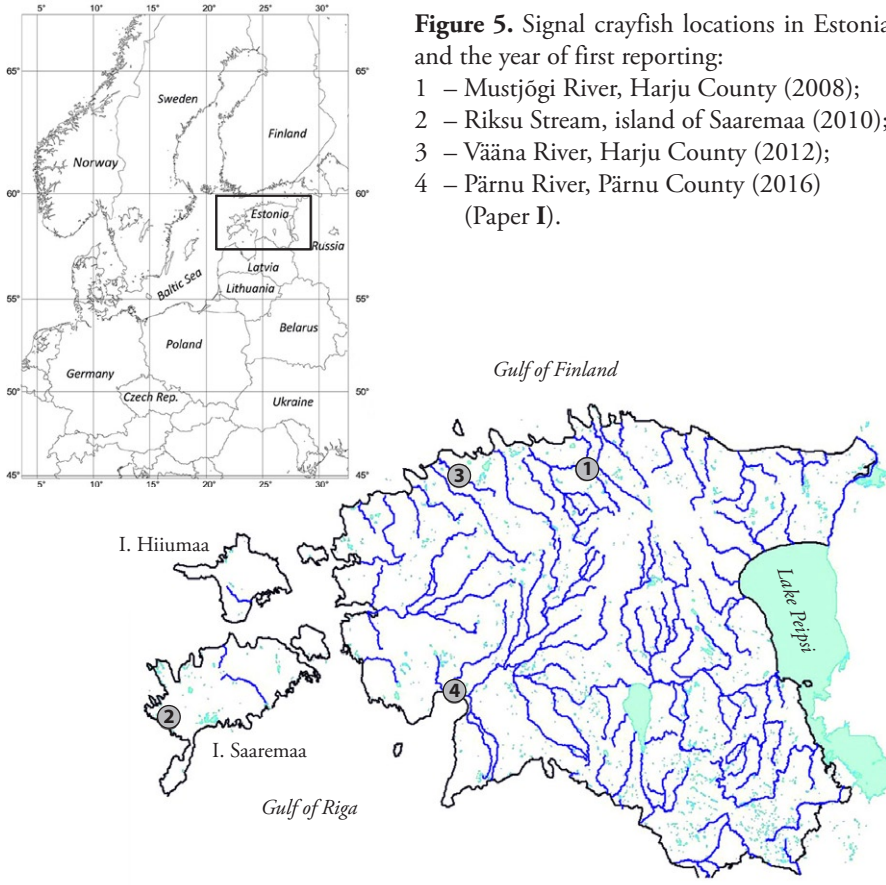

Figure 5. Signal crayfish locations in Estonia and the year of first reporting:
1 – Mustjõgi River, Harju County (2008);
2 – Riksu Stream, island of Saaremaa (2010);
3 – Vääna River, Harju County (2012);
4 – Pärnu River, Pärnu County (2016) (Paper **I**).

detection of signal crayfish was obtained from a database of standardized test fishings and crayfish stocking of the Chair of Aquaculture of EULS, which has been annually updated since 2003 and includes data back to 1989.

4.2. Detection of crayfish plague (I)

To confirm the crayfish plague occurrence in Estonia, samples from 16 populations with mass mortalities or from cage experiments were analysed for the presence of *A. astaci* using molecular genetic tools (Table 3).

4.2.1. Molecular tests for plague detection

Molecular tests for crayfish plague detection were conducted in four different laboratories. Analyses at the Norwegian Veterinary Institute (2007), EULS (2010 and 2011 by Liisi Sepping and Riho Gross) and the University of Poitiers (2014–2016) were based on the real-time qPCR methodology of Vrålstad *et al*. (2009). Analyses at the Finnish Food Safety Authority (2009 and 2010) were based on the PCR methodology described by Oidtmann *et al*. (2004).

Here I describe briefly the methods and protocols used by myself during my stay in the University of Poitiers. Samples were stored in 96% ethanol at room temperature, and were analysed as described by Collas *et al*. (2016) study. Three sub-samples were dissected per individual crayfish. Crayfish tissue from the soft abdominal cuticle, one uropod and (if present) melanised spots on the exoskeleton were dissected from each crayfish using sterile instruments. Tissues from each individual were placed in a single Eppendorf 1.5 mL tube in 96% ethanol and stored until analysis. For analysis, tissue samples were cut into small pieces, placed in a 2 mL Eppendorf tube and dried with an open lid in a heating-block at 70 °C for ~10–12 min. Before further processing, 360 µL of ATLBuffer from the DNeasy tissue kit (Qiagen) and 40 µL of proteinase K solution were added to the dissected tissue. The mixture was then crushed with one scoop (ca. 50 µL) of stainless steel beads (1.6 mm diameter) using a BBX24B Bullet Blender (Next Advance) for 10 min at maximum speed. The amount of 400 µL of the Buffer AL was added to DNA extractions from the crushed cuticle, then the rest of the spin-column protocol of the DNeasy tissue kit was followed.

The isolated material was then tested for the presence of *A. astaci* by the quantitative TaqMan® MGB real-time PCR developed by Vrålstad *et al*. (2009), using the LightCycler 480 Instrument (Roche). Experimental procedures were identical to those used by Filipova *et al*. (2013). Based on their PCR forming units (PFU) values, samples were classified into semi-quantitative categories of pathogen load, ranging from A0 (no traces of A. astaci DNA) to A7 (extremely high amount of *A. astaci* DNA in the sample), as proposed by Vrålstad *et al*. (2009).

4.2.2. *A. astaci* genotype determination

Nine microsatellite loci (Aast 2, 4, 6, 7, 9, 10, 12, 13, 14), developed by Grandjean *et al*. (2014) for *A. astaci*, were genotyped on four noble crayfish samples that were positive for *A. astaci* presence from the qPCR analysis, with a level of infection of at least A3 according to the agent level categories described by Vrålstad *et al*. (2009). All experimental procedures for microsatellite genotyping were identical to those described by Grandjean *et al*. (2014).

4.3. Low temperature experiment with marbled crayfish (II)

To examine the low temperature water tolerance of marbled crayfish, and the possibility for their invasion into northern Europe (paper **II),** the survival, growth, behaviour and reproduction of marbled crayfish at low temperature were examined in the winter period, from September 9, 2011 to April 18, 2012 (110 days) in the outdoor tanks at the Chair of Aquaculture of EULS. In general, low temperature water tolerance depends on body size, so the survival of two body size groups of marbled crayfish were compared.

The sampled marbled crayfish were bought from an Estonian aquarium shop, and stocked into the aquarium at the Chair of Aquaculture of EULS. They were reared in two 1 m^3 outdoor tanks. Twenty-five larger animals, with a mean total length (TL) 42.2 mm ± 6.4 SD and mean weight 2.1 g ± 0.4 SD were stocked into Tank 1. And 25 smaller animals, with a mean TL 31.6 mm ± 2.8 SD and mean weight 0.9 g ± 0.2 SD were stocked into Tank 2. At the start and end of the experiment, the TL, weight and number of eggs of each individual were recorded. Dead animals or moulting individuals were recorded daily during the

experiment. To make the water temperature in the experimental tanks similar to natural water systems in Estonia, the water temperature during the 2011/2012 winter season at Piusa River Estonia was recorded, and the water temperatures in the experimental tanks were regulated using electric heaters.

4.4. Experimental setup of feeding trial of marbled crayfish (III)

The feeding trial in paper **III** was set up at the Laboratory of the Chair of Aquaculture of EULS and lasted for 123 days (from February 8 to June 10, 2013). Approximately 19-week-old marbled crayfish were all collected from progeny of a single parent. The initial size of crayfish varied from 13 to 28 mm TL (from 0.08 to 0.46 g), and there were four carapace colour variants (light blue, blue, light brown, dark brown) were observed.

A total of 90 crayfish were randomly allocated into six 55 L glass aquaria in triplicates per treatment (45 crayfish per treatment, 15 crayfish per aquarium) and were kept under the same rearing conditions. Each aquarium was equipped with a filter and aerator, black coloured gravel substrate on the bottom of the tank and 15 pieces of PVC pipes that served as shelters for the crayfish. The water temperature varied from 20 to 22 ^{o}C according to room temperature. The photoperiod was adjusted to the natural light regime. Aquaria were cleaned once per week.

The crayfish were divided into two groups. Group 1 crayfish were fed with a common carp feed without astaxanthin, and the crayfish in Group 2 were fed with a commercial discus feed with astaxanthin (20% shrimp) at a quantity of 5% of crayfish body weight once per day, except for weekends.

The crayfish were individually weighed, measured and examined for the presence of eggs at the beginning (February 8, 2013), middle (April 11, 2013) and at the end (June 10, 2013) of the feeding trial. Carapace colour was examined visually in a plastic box (12.8 × 9.2 × 4.0 cm) through the water with a white paper sheet as a background. Altogether eight colour variants could be discriminated: light blue, blue, dark blue, light brown, dark brown, light grey, dark grey, and greyish brown (Table 1 in paper **III**).

4.5. Development and characterization of novel tetranucleotide microsatellite markers (IV)

4.5.1. Genomic library preparation and sequencing

Genomic DNA was isolated from the leg muscle tissue of three adult specimens of *A. astacus* (collected from Lake Pangodi, Estonia), using the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Germany). *Hin*fI restriction enzyme digested and purified genomic DNA was prepared for sequencing using the ClaSeek Library Preparation Kit (Thermo Fisher Scientific Inc, USA) and the Illumina TruSeq DNA LT Sample Prep kit (Illumina, San Diego, USA). The libraries were quantified with the Qubit 2.0 Fluorometer (Invitrogen, Grand Island, USA) and TapeStation 2200 (Agilent Technologies, Santa Clara, USA), validated by qPCR using the Kapa Library Quantification Kit (Kapa Biosystems, Woburn, USA) and sequenced on the Illumina MiSeq flowcell with 2 × 300 bp paired-end reads using the MiSeq Reagent Kit v3 (Illumina, San Diego, USA). Paired-end reads were stitched with PEAR using default settings (Zhang *et al*. 2014).

4.5.2. Detection of microsatellite motifs and initial testing of loci for amplification success and polymorphism in *A. astacus* and *A. leptodactylus*

For microsatellite motif detection, sequence selection and PCR primer design software QDD version 3.1 was used with default settings (Meglécz *et al*. 2014). All sequences from the three specimens that contained tetranucleotide repeats were pooled and subjected to PCR primer design. A total of 48 primer pairs were selected, according to the criteria of Meglécz *et al*. (2014). These were initially tested for amplification success and polymorphism in eight individuals of *A. astacus* (four individuals from water reservoirs Boskovice & Landštejn in the Czech Republic, the Danube-Black Sea catchment and one individual from each of the four different wild populations in Estonia, the Baltic Sea catchment) and also in eight individuals of *A. leptodactylus* (four individuals each from Korana and Mrežnica populations in Croatia). Forward primers were designed with a 19 bp M13 tail that was labelled during the PCR reaction using a universal fluorecently (either 6-FAM, NED, PET or VIC) labelled M13 primer (CACGACGTTGTAAAACGAC). The PCR reaction (10 µl) contained 1x Type-it Multiplex PCR Master Mix (QIAGEN,

Germany), 100 nM of forward primer, 200 nM of M13 and reverse primer, and ~50 ng of DNA template. The touchdown program was used for PCR amplification: initial activation of 5 min at 95 °C, followed by 20 cycles of 30 sec at 95 °C, 90 sec at 60 °C, 30 sec at 72 °C, with the annealing temperature decreasing at 0.5 °C per cycle. This was followed by 10 cycles of 30 sec at 95 °C, 90 sec at 50 °C, 30 sec at 72 °C and a final extension for 30 min at 60 °C. Electrophoresis was performed using the Applied Biosystems 3500 Genetic Analyser (Life Technologies, USA) and the length of fragments was determined using internal GeneScan 600 LIZ Size Standard v2.0 (Life Technologies, USA) and GeneMapper 5 software (Life Technologies, USA).

4.6. Statistical analyses

In paper **II**, the relationship between water temperature and survival of marbled crayfish in Estonia in the 2011/2012 winter season is presented by the mean water temperatures monthly with standard deviations and the survival number of marbled crayfish. Excel was used to perform calculations.

In paper **III**, the effect of feed on growth was estimated by the Student's t test, the effect of feed on colouration was estimated by the Fisher's exact test, and the effect of feed on mortality was estimated by the odds ratio test. The effect of aquaria on growth within the treatment groups was estimated by the Tukey multiple comparisons test. All data were tested for normality prior to analysis. Statistical analyses were conducted using R Statistical Software package 3.0.2.

5. RESULTS

5.1. Distribution of signal crayfish in Estonia and dynamics of its populations (I)

Signal crayfish have now been found in four sites in Estonia (Table 2; Fig. 5). The first specimen of signal crayfish was caught in Mustjõgi River, Harju County in 2008 (Fig. 5). The noble crayfish population had been extinct there, but had recovered after restocking in 1997–1999. Test fishings in 2005 showed the presence of noble crayfish (CPUE 1.2), but after the identification of signal crayfish in 2008, noble crayfish were not found except for a single specimen in 2014. No signal or noble crayfish were found during extensive test fishings in Mustjõgi River and its tributaries in 2009 (Table 2). In 2010–2012 a few signal crayfish were found at the same site, in a 25–30 m long section of the river (Table 2). During the years 2013–2016 no signal crayfish were caught in test fishings.

The second signal crayfish population was found in 2010, on the island of Saaremaa in Riksu Stream. Additional test fishing with an increased number of trap nights was carried out, and 61 (CPUE 0.12) signal crayfish were caught from a 500 m section downstream of the previous site in Riksu Stream (Table 2). Noble crayfish were not found at that site, although in the early 2000s there was an extant noble crayfish population. About 50 noble crayfish were caught per 10 m in 2002 (unpublished data from EULS). In 2011 and 2012 a new site was found just upstream, but the number of signal crayfish in the total catch was lower than in 2010, despite an increase in the number of trap nights (Table 2). In 2013, new signal crayfish sites were found further upstream, and up to 2014 signal crayfish were known to be present in an approximately three km section of Riksu Stream. The total catch in 2013–2016 had increased (CPUE 0.19 up to 3.31) compared to the years 2010–2012 (Table 2). The estimated migration rate of signal crayfish in Riksu Stream during the last two years has been one km upstream per year.

The third signal crayfish population was found in 2012 in Vääna River, Harju County. At the end of the 1990s there was a dense noble Crayfish population (CPUE 4.8). The situation in Vääna River is different from other sites because both noble crayfish and signal crayfish are living in sympatry. In 2012 one noble crayfish and one signal crayfish were found

Table 2. The number of trap nights, caught individuals of signal crayfish *Pacifastacus leniusculus* (*P.l.*) and noble crayfish *Astacus astacus* (*A.a.*) and total CPUE of test fishings during 2008–2016 (Paper **I**)

Watercourse / coordinates	**2008**	**2009**	**2010**	**2011**	**2012**	**2013**	**2014**	**2015**	**2016**
Mustjõgi River (Harju county) 25° 36'48" E[1] 59° 17'50" N[1]									
No of traps	40	80	40	340	200	310	100	80	40
No of *P.l.*	1	0	5	7	1	0	0	0	0
No of *A.a.*	0	0	0	0	0	0	1	0	5
CPUE	0.03	0	0.13	0.02	0.01	0	0.01	0	0.13
Riksu Stream (Island of Saaremaa) 22° 05'13" E[2] 58° 11'39" N[2]									
No of traps	–	–	505	1,400	440	734	238	514	310
No of *P.l.*	–	–	61	50	31	140	256	743	1,027
No of *A.a.*	–	–	0	0	0	0	0	0	0
CPUE		–	0.12	0.04	0.07	0.19	0.81	1.45	3.31
Vääna River (Harju county) 24° 39'26" E[1] 59° 18'59" N[1]									
No of traps	–	–	–	–	135	300	160	180	180
No of *P.l.*	–	–	–	–	1	1	11	4	2
No of *A.a.*	–	–	–	–	1	18	22	5	1
CPUE	–	–	–	–	0.01	0.06	0.21	0.05	0.02
Pärnu River (Pärnu county) 24° 29'30" E[3] 58° 23'12" N[3]									
No of traps	–	–	–	–	–	–	–	–	100
No of *P.l.*	–	–	–	–	–	–	–	–	16
No of *A.a.*	–	–	–	–	–	–	–	–	1
CPUE	–	–	–	–	–	–	–	–	0.17

[1]given coordinates are midpoint of the trap line
[2]signal crayfish distribution area is 3 km upstream from the given coordinates
[3]after additional test fishing signal crayfish were found in two other locations in Pärnu River (24° 28'53" E, 52° 23'18" N and 24° 30'02" E, 58° 23'18" N).

in 135 trap nights (Table 2). In 2013, test fishing included more traps (300 trap nights) and one signal crayfish was found. Noble crayfish dominated in catch. In 2014, the abundance of both species had increased. In 2015 and 2016 fewer signal and noble crayfish were caught using the same number of traps (Table 2).

The fourth signal crayfish population was found in 2016 by local fishermen in Pärnu Bay, 2 km from the coast, and in the mouth of Pärnu River in the middle of Pärnu City (Pärnu County). In a subsequent test fishing,

16 signal crayfish and one noble crayfish were caught from the river over 100 trap nights (Table 2; Fig. 5).

5.2. Crayfish plague events in Estonia during 2006–2015 and their connection to signal crayfish distribution (I)

Crayfish plague was detected by qPCR in all of the analysed sites. Because the majority of samples had low levels of *A. astaci* DNA, multilocus genotype group of *A. astaci* was determined only in the case of four crayfish mass mortalities. A sample from Laugi Stream (2007) on the island of Saaremaa showed *A. astaci* multilocus genotype SSR-E, and a sample from Pärlijõgi River (2010) in Võru County showed *A. astaci* multilocus genotype SSR-B. The other two cases in Härjanurme fish and crayfish farm (2010) in Jõgeva County and Avijõgi River (2015) in East-Viru County had *A. astaci* multilocus genotype SSR-A (Table 3). Crayfish plague analyses in signal crayfish watercourses: Mustjõgi River, Riksu Stream and Vääna River, indicated the presence of *A. astaci* (Table 3) but the genotype could not be determined.

5.3. Effect of temperature on marbled crayfish (II)

5.3.1. Effect of temperature on survival rate

At the start of the trial the average water temperature was 13.8 °C in September 2011. After October 10, 2011, the water temperature decreased to below 10 °C for a five month period. December, January and February were the coldest months, when the average water temperatures were below 5 °C in both tanks (Fig. 6).

The first dead crayfish was found on November 21, 2011 in Tank 1 with the larger crayfish, when the water temperature decreased to 0.7 °C. Before January 26, 2012, there were no dead animals in Tank 2, but the mortality increased rapidly when the water temperature decreased to less than 2 °C and the bottom of tank was iced for 16 days. Sixteen marbled crayfish died during this period. Mortality in Tank 1 increased when the water temperature increased rapidly at the end of February. At the end of the experiment, the survival rate in Tank 1 was higher (60%) than in Tank 2 (8%) (Fig. 6).

Table 3. Crayfish plague events in Estonia during 2006–2015 (Paper **I**)

Sample origin[a]	Crayfish species[b]	Sampling year	No. and status of analysed individuals[c]	PCR/ qPCR result[d]	Highest agent level[e]	Genotype group[f]	Analysis site[g]	Analysis year
Pihtla crayfish farm, Island Saaremaa, OB in 2006	*A.a*	2006	2 alive*	2 posit	A3	NT	NVI	2007
		2006	2 alive	2 posit	A2	NT	Univ-Poitiers	2016
Pähkla fish and crayfish farm, Island Saaremaa, OB in 2007	*A.a.*	2007	1 dead*	1 posit	A7	NT	NVI	2007
Põduste River, Island Saaremaa, OB in 2007, CE in 2008	*A.a.*	2007	11 dead*	8 posit	A7	NT	NVI	2007
		2008	1 alive	1 posit	A1	NT	Univ-Poitiers	2016
Laugi Stream, Island Saaremaa, OB in 2007	*A.a.*	2007	4 alive*	2 posit	A6	NT	NVI	2007
		2007	1 dead	1 posit	A3*	E	Univ-Poitiers	2016
Sõrve crayfish farm, Island Saaremaa, OB in 2007	*A.a.*	2007	2 alive*	1 posit	A2	NT	NVI	2007
Riksu Stream, Island Saaremaa	*P.l.*	2011	4 alive	3 posit	NK	NT	EULS	2011
		2011	2 alive**	2 posit	A3	NT	Univ-Poitiers	2014
		2013	5 alive	1 posit	A2	NT	Univ-Poitiers	2014
		2015	6 alive	4 posit	A2	NT	Univ-Poitiers	2015
Masa River, Island Saaremaa, OB in 2013	*A.a.*	2013	4 dead	1 posit	A2	NT	Univ-Poitiers	2014
		2013	1 alive	1 posit	A2	NT	Univ-Poitiers	2016
Leevi River, Põlva, OB in 2007, CE in 2009	*A.a.*	2007	2 dead*	2 posit	A5	NT	NVI	2007
		2007	2 dead	2 neg	A0	NT	Univ-Poitiers	2016
		2009	1 died*, 1 alive*	2 posit*	NK	NT	EVIRA	2009
Mustjõgi River, Võru, DD in 2008	*A.a.*	2008	1 alive*	1 posit*	NK	NT	EVIRA	2009

Mustjõgi River, Harju, CE in 2009	*A.a.*	2009	2 dead*	2 posit*	NK	NT	EVIRA	2009
	P.l.	2009	1 alive*	1 neg*	NK	NT	EVIRA	2009
Lake Selja, West-Viru, OB in 2009	*A.a.*	2009	3 dead	3 posit*	NK	NT	EVIRA	2009
		2009	2 dead	2 posit	A1	NT	Univ-Poitiers	2016
Pärlijõgi River, Võru, OB in 2008-2010	*A.a.*	2010	4 dead	3 posit*	NK	NT	EVIRA	2010
		2010	1 dead	1 posit	NK	NT	EULS	2010
		2010	1 dead	1 posit	A4*	B	Univ-Poitiers	2014
		2010	2 dead	2 posit	A5*	B	Univ-Poitiers	2016
Härjanurme fish and crayfish farm, Jõgeva, OB in 2010	*A.a.*	2010	1 dead	1 posit	NK	NT	EULS	2010
		2010	1 dead	1 posit	A4*	A	Univ-Poitiers	2016
Taebla River, West, DD in 2010	*A.a.*	2011	1 alive	1 posit	NK	NT	EULS	2011
		2011	1 alive**	1 posit	A3	NT	Univ-Poitiers	2014
Vääna River, Harju	*P.l.*	2015	4 alive	2 posit	A1	NT	Univ-Poitiers	2015
	A.a.	2015	2 alive	2 neg	A0	NT	Univ-Poitiers	2015
Avijõgi River, East-Viru, OB in 2015	*A.a.*	2015	3 dead	3 posit	A4*	A	Univ-Poitiers	2015

[a] Sample origin: All samples originate from suspected incidences of crayfish plague in Estonia. The sample origin listed includes the name of population (farm, lake, river or stream) and county. The crayfish samples originate either outbreak (OB), decline of density (DD) or cage experiment (CE) that has been used for crayfish plague surveillance in the years after an outbreak.

[b] *A.a. = Astacus astacus; P.l. = Pacifastacus leniusculus.*

[c]*Crayfish tissue material or DNA-isolates were not stored, ** DNA used in analyses was extracted at EULS in 2011.

[d] *Analyses at the Finnish Food Safety Authority (EVIRA) based on the Oidtmann *et al.* (2004) PCR methodology. Others based on the Vrålstad *et al.* (2009) qPCR methodology.

[e]Only the highest detected agent level is given. Results marked * have been confirmed by sequencing. NK = not known – the agent level was not included in the protocol.

[f]Genotype group determined based on microsatellite markers. NT = not tested.

[g]NVI = Norwegian Veterinary Institute; EVIRA; Univ-Poitiers = University of Poitiers; EULS.

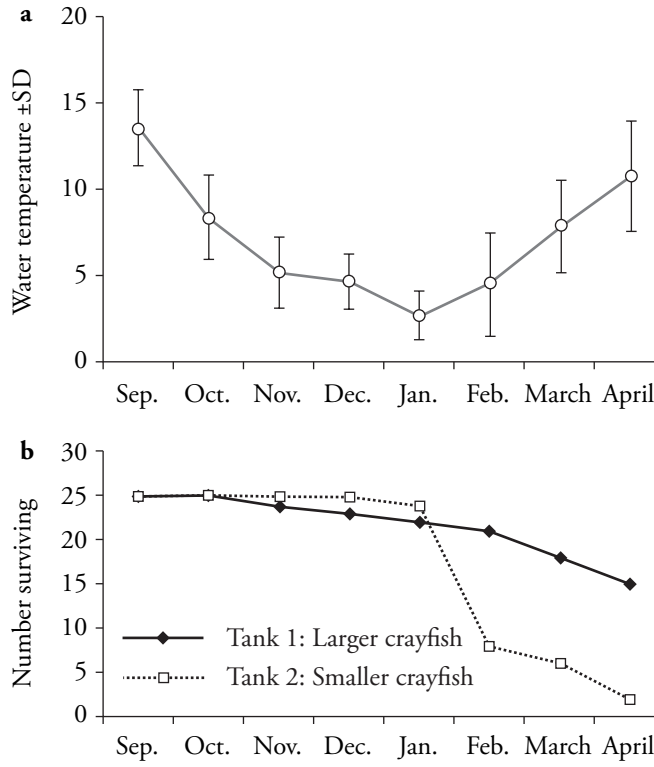

Figure 6. Relationship between water temperature and survival of marbled crayfish in the 2011/2012 winter season (Paper **II**).

5.3.2. Effect of temperature on growth, reproduction and behaviour

During the experiment, two crayfish moulted at temperatures below 10 °C. The first was on November 9, 2011, when the water temperature had been below 10 °C in Tank 2. Moults in Tank 1 were not observed. At the start of the experiment, five marbled crayfish stocked into Tank 1 carried eggs. No crayfish with eggs were stocked in Tank 2. The first dead crayfish with eggs was found on January 3, 2012. All the eggs were coloured orange and covered with mould. The four remaining egg-bearing crayfish could later not be identified, probably because the eggs were lost. Hatchlings were not observed. At the end of the experiment, no crayfish had eggs. When the surviving crayfish were transferred back into the indoor aquarium at a temperature of approximately 20 °C, one spawned eggs

within the first week, and the other 15 spawned soon after. These eggs hatched normally and their progeny survived.

Marbled crayfish were active and ate at temperatures above 10 °C. Crayfish became less active when the water temperature decreased, and stopped eating below 10 °C. Most crayfish hid in shelters at temperatures below 5 °C. At temperatures below 2 °C, some marbled crayfish came out of the shelters and lay motionless on the bottom of tank appearing as if dead, but movement could be seen when they were observed closely. When the water temperature was then increased above 4 °C, the crayfish became more active and the shelters were used less often. One crayfish had a missing claw and another had a claw in regeneration, suggesting the occurrence of aggressive behaviour.

5.4. Effects of different feeds on rearing of marbled crayfish (III)

At the beginning of the trial, the marbled crayfish had mostly light or darker brown and light blue and blue carapace colourations. After two months of feeding, the crayfish fed with astaxanthin-free carp feed (Group 1) were coloured much lighter and more variably than the crayfish fed with the astaxanthin-rich discus feed (Group 2), which exhibited almost uniformly the same blue carapace colouration. Only three crayfish in Group 2 exhibited the darker brown colouration. At the end of the trial, all the crayfish in Group 2 exhibited the same dark blue carapace colouration, while the crayfish in Group 1 exhibited more variable colouration in colour shades of grey. The differences between the feeding groups in carapace colouration were statistically significant ($P < 0.05$), both at the middle and at the end of the feeding trial.

At the beginning of the experiment, there were no significant differences in the mean body weight and total length between the two experimental groups (Fig. 7). However, two months later, the crayfish in Group 2 (discus feed) were significantly larger ($P < 0.05$) than the crayfish in Group 1 (carp feed), and by the end of the trial the differences between the two groups were even greater (Fig. 7). During the whole feeding trial (123 days), the mean body length of Groups 1 and 2 increased by 15.9–24.3 mm, respectively, and the mean body weights increased by 0.97–2.24 g, respectively. The effect of aquaria on growth was not significant within both treatment groups ($P > 0.05$).

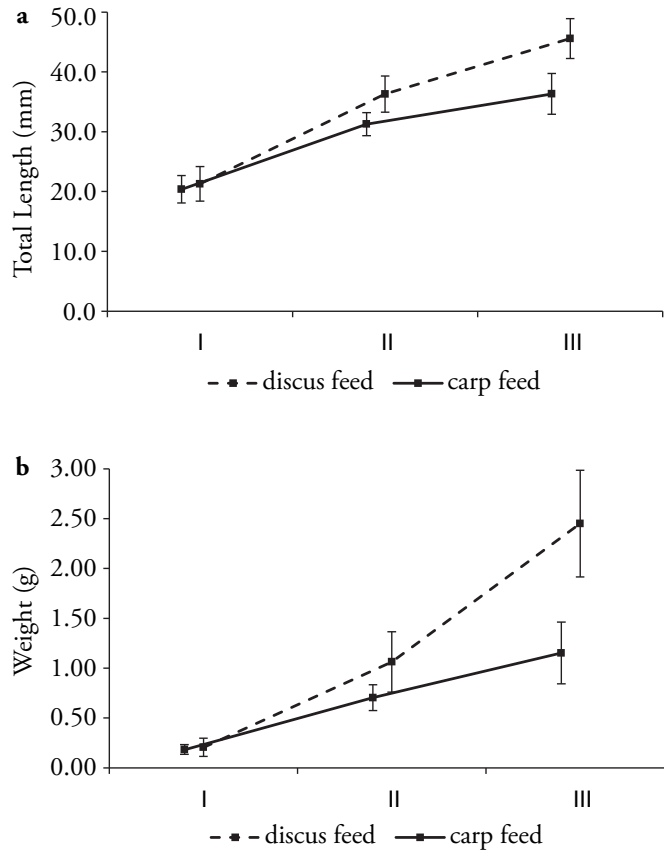

Figure 7. Mean TL (a) and weight (b) ± SD of marbled crayfish at the beginning, middle and the end of the feeding trial (Paper **III**).

At the beginning of the trial, no crayfish with eggs were observed. In the middle of the trial, three marbled crayfish in Group 1 (carp feed) had eggs, in contrast to crayfish in Group 2 (discus feed) who had no eggs. At the end of the trial, six crayfish in both feeding groups (15% in Group 1 and 17% in Group 2) had eggs. This indicates that the type of feed had no effect on the maturation of marbled crayfish.

After two months of the feeding trial, the mortality of marbled crayfish in Groups 1 and Group 2 were 7 and 11%, respectively. At the end of the feeding trial, the mortality among crayfish fed with discus feed (Group 2)

was twice as high (22%) as the mortality among the crayfish fed with carp feed (Group 1, 11%). However, the difference was statistically not significant (odds ratio 0.5 (CI 0.156–1.604)).

5.5. Development of tetranucleotide microsatellite loci in *A. astacus* and cross-species amplification in *A. leptodactylus* (IV)

A total of 13,490,148 paired-end reads were obtained using the Illumina MiSeq System and 5,686 reads (0.042%) contained tetranucleotide repeats. Of the total of 48 loci that were selected for initial testing, 35 amplified successfully and 25 were polymorphic among eight individuals of *A. astacus* (Table 4).

The 48 loci were also tested on eight individuals of *A. leptodactylus* from Croatia. Of these, 21 loci cross-amplified successfully, of which 14 were polymorphic, possessing from two to five alleles (Table 4). The usefulness of these polymorphic loci for genetic studies of *A. leptodactylus* should be tested on larger sample sizes.

In addition, 13 loci (both monomorphic and polymorphic) possessed species-specific allele size range in *A. astacus* and *A. leptodactylus* and can be potentially used for the detection and confirmation of possible hybrids between these sister species (Table 4).

Table 4. Characterization of tetranucleotide microsatellite loci in eight individuals of *A. astacus* and *A. leptodactylus*. Loci shown are only those successfully amplified in one or both species (Paper **IV**)

Locus	Repeat motif	Label of the M13 primer	*A. astacus*			*A. leptodactylus*		Suitability for hybrid detection	GenBank accession no.
			No. of alleles	Expected allele size (bp)*	Observed allele size range (bp)*	No. of alleles	Observed allele size range (bp)*		
Aast4_2	ACCT	6FAM	2	115	106 - 110	2	176 - 184	yes	KU955535
Aast4_3	ACGC	6FAM	2	120	115 - 119	4	160 - 167	yes	KU955536
Aast4_5	AATG	6FAM	1	141	137	2	121 - 125	yes	KU955538
Aast4_7	ACAT	6FAM	2	159	156 - 168	1	158	no	KU955540
Aast4_8	ACAG	6FAM	1	164	163	2	129 - 133	yes	KU955541
Aast4_10	ACAT	6FAM	5	181	165 - 189	4	140 - 177	no	KU955543
Aast4_12	AATG	6FAM	1	189	187	2	171 - 175	yes	KU955545
Aast4_14	ACAT	VIC	1	191	192	0	No amp.	no	KU955547
Aast4_16	ACGC	VIC	2	207	199 - 203	3	176 - 182	yes	KU955549
Aast4_17	ACGC	VIC	4	207	190 - 233	0	No amp.	no	KU955550
Aast4_18	ACAG	VIC	3	209	206 - 214	2	207 - 211	no	KU955551
Aast4_19	ACAT	VIC	3	209	205 - 213	0	No amp.	no	KU955552
Aast4_20	AGCC	VIC	3	209	193 - 205	5	166 - 268	no	KU955553
Aast4_22	AAGG	VIC	2	213	211 - 215	1	211	no	KU955555
Aast4_23	AGAT	VIC	0	213	No amp.	1	122	no	KU955556
Aast4_24	AGGC	VIC	4	213	210 - 226	1	308	yes	KU955557
Aast4_25	ACAG	NED	1	217	217	0	No amp.	no	KU955558

Aast4_26	AGGC	NED	3	224	220 - 232	2	249 - 258	yes	KU955559
Aast4_27	ACGC	NED	1	225	306	0	No amp.	no	KU955560
Aast4_30	ACCT	NED	5	253	238 - 254	2	144 - 152	yes	KU955563
Aast4_31	AGCC	NED	1	254	250	0	No amp.	no	KU955564
Aast4_32	ACAT	NED	2	256	255 - 259	1	234	yes	KU955565
Aast4_33	AGGC	NED	0	259	No amp.	4	355 - 379	no	KU955566
Aast4_34	ACTC	NED	3	259	252 - 260	1	240	yes	KU955567
Aast4_35	ACAG	NED	2	260	258 - 262	0	No amp.	no	KU955568
Aast4_37	ACAT	PET	2	265	266 - 270	0	No amp.	no	KU955570
Aast4_38	ACAT	PET	2	266	268 - 276	0	No amp.	no	KU955571
Aast4_39	ACCT	PET	3	271	271 - 316	0	No amp.	no	KU955572
Aast4_40	ACAT	PET	1	274	274	1	241	yes	KU955573
Aast4_41	ACGC	PET	1	277	279	0	No amp.	no	KU955574
Aast4_42	AGGC	PET	3	279	270 - 282	0	No amp.	no	KU955575
Aast4_43	ACAT	PET	2	280	276 - 371	3	300 - 308	no	KU955576
Aast4_44	ACAG	PET	4	310	311 - 335	0	No amp.	no	KU955577
Aast4_46	ACAT	PET	3	310	299 - 307	0	No amp.	no	KU955579
Aast4_47	ACGC	PET	2	315	316 - 320	0	No amp.	no	KU955580
Aast4_48	ACAT	PET	2	319	311 - 319	3	287 - 295	yes	KU955581

*with 19 bp M13-tail (CACGACGTTGTAAAACGAC)

6. DISCUSSION

6.1. Status and distribution of signal crayfish in Estonia and its role in a series of plague outbreaks which have taken place recently in noble crayfish populations

The spread of NICS is a combination of natural expansion and human-assisted introductions, which may be either deliberate or accidental (Peay & Füreder 2011). In the Estonian case, natural migration is not an issue, since the discovered locations are not connected to the waters inhabited by the species in neighbouring countries. In addition, there is no direct connection between the signal crayfish-inhabited rivers in Estonia. The main vector for the introduction of NICS into Estonia may be the international trade in live crayfish and the threat factor is the increased interest of crayfish farmers in the introduction of alien species (Paaver & Hurt 2009). Losses of noble crayfish due to crayfish plague outbreaks, together with information about the efficient and cheap exploitation of signal crayfish populations in Finland has increased the interest of crayfish farmers and owners of water bodies in introducing crayfish plague-resistant crayfish species. The threat to the noble crayfish from illegal introductions, catching, trade and farming of NICS (Paaver & Hurt 2009) ensures a need for improved legislation restricting the spread of NICS. These changes were included into the Estonian Nature Conservation Act which was adopted in 2004.

This study showed that the crayfish plague was detected for the first time on the island of Saaremaa in 2006, which was four years earlier than signal crayfish were found there. Crayfish plague caused a collapse of the stocks in the three crayfish farms during 2006–2007, and in two wild populations, in rivers that were connected to the Pähkla fish and crayfish farm on the island of Saaremaa (Table 3; **I**). Analysis of one sample from 2007 from the Laugi Stream (near to the crayfish farm) outbreak showed *A. astaci* multilocus genotype SSR-E, which was originally isolated from the spiny-cheek crayfish (Kozubíková *et al*. 2011). Based on these data it may be assumed that at least the occurrence of crayfish plague genotype group E on the island of Saaremaa could be linked to the trade with live crayfish in Lithuania. *O. limosus* occurrence has been confirmed in Latvia (Briede 2011) and Lithuania (Arbăciauskas *et al*. 2011) and in autumn 2017 the first specimens of *O. limosus* were also found in Esto-

nia (unpublished data from EULS). *Aphanomyces astaci* could spread to the island of Saaremaa by the careless transfer of contaminated crayfish traps or live fish, and crayfish transport. The presence of genotype group A in the case of mass mortalities in Härjanurme fish and crayfish farm (2010) and Avijõgi River (2015) and the genotype group B (strain originating from *P. leniusculus*) in Pärlijõgi River (2010) was not surprising. Recently, Vrålstad *et al*. (2014) and Maguire *et al*. (2016) reported that both genotype groups were responsible of a large series of outbreaks in noble crayfish stock in Norway and Croatia, respectively. In Pärlijõgi River the signal crayfish has never been detected. This river has had a moderate noble crayfish population density (CPUE 3.0) in the middle years of the 2000s. A crayfish plague outbreak was detected in 2010 and *A. astaci* genotype group analysis revealed the B strain (Table 3; **I**). Pärlijõgi River belongs to the Gauja River watershed in Estonia and Latvia, but from the data presented by Briede (2011), signal crayfish do not inhabit that river. However, the possibility cannot be excluded that other animals (e.g. semiaquatic mammals or birds) can also spread the spores (Makkonen *et al*. 2013).

There is no information on how signal crayfish spread to Estonia, but this may have already happened before 2008. No evidence about the legal introductions of NICS to Estonia has been found. Strict regulations on the import of live crayfish to certain European countries (Peay 2009) and intensive proactive conservation measures to protect the ICS (Holdich *et al*. 2009) have not been sufficient to reduce the trade and spread of NICS (Peay 2009). The trade in ornamental freshwater crayfish has grown rapidly in the last decade, and many aquarium shops in Estonia have sold marbled crayfish that are able to survive in North European countries and transmit the crayfish plague (Keller *et al*. 2014; Kaldre *et al*. 2015; Mrugała *et al*. 2015).

The data reported here indicate different patterns of development of the introduced NICS populations. So far, signal crayfish have been responsible for the disappearance of the noble crayfish population in Estonia at least in two sites – Mustjõgi River and Riksu Stream. In the Mustjõgi River noble crayfish have not been found after the discovery of signal crayfish, but today, the signal crayfish population also seems to have been lost. The last signal crayfish in Mustjõgi River was seen in 2012. During 2015 and 2016 1,000 noble crayfish in total have been restocked into Mustjõgi River, and have so far survived. Five noble crayfish (CPUE 0.1)

were caught in 2016 from the same site where the signal crayfish was earlier detected. Signal crayfish were probably brought to the island of Saaremaa in 2004–2005, and were responsible for the disappearance of the noble crayfish population in Riksu Stream in the beginning of the 2000s. First detected in 2010, during the last six years the signal crayfish population has been growing. There are no obstacles to its spread in both directions – either downstream (via Riksu Lake towards the sea) or upstream. Until 2013, there was a plan to dry out a part of the river inhabited by signal crayfish, by constructing a new side channel. However, new sites with signal crayfish were found upstream, making this activity senseless, making possible eradication almost impossible. The case of Pärnu bay also shows that signal crayfish can tolerate brackish (salinity 2–3 ppt) conditions and spread via coastal areas.

Noble crayfish populations did not disappear from all of the signal crayfish locations. Although signal crayfish are chronic carriers of *A. astaci,* and are also capable of occupying habitats of European ICS, having wider environmental tolerance (Peay & Füreder 2011), in Estonia noble crayfish still persist in sympatry with signal crayfish in two localities, in Vääna and Pärnu Rivers. Signal crayfish were found together with noble crayfish in a small (200 m) site in Vääna River in 2012, and test fishing in each subsequent year showed the presence of both species at the same site (Table 2; **I**). Crayfish plague analyses of signal crayfish showed a low level of *A. astaci*, but noble crayfish were not infected (Table 3; **I**). The number of analysed specimens was small, and the period of the study short, and it is suggested that monitoring including more individuals and over a longer time should be carried out. Nevertheless, this study confirmed that permanent coexistence between ICS and NICS is possible, as has also been described in other studies (Schrimpf *et al*. 2013; James *et al*. 2017).

6.2. A threat of a new non-indigenous crayfish species – parthenogenetic marbled crayfish to Estonian noble crayfish

6.2.1. Effect of temperature on survival rate

The experiment in Paper **II** indicated that marbled crayfish could survive for a short period (less than one week) at 1–2 °C, but longer periods (more than two weeks) at low temperatures (1–2 °C) caused high mortality (Fig. 6; **II**). The differences in mortality could have been caused by the

different sizes of the crayfish in the tanks, as the larger crayfish seemed to be more tolerant of very low temperatures (Fig. 6; **II**). However, both tanks went through three months at an average water temperature below 5 °C, and marbled crayfish survived those harsh conditions. Water temperature varies temporally on a daily and annual cycle in nature, but water temperatures do not change rapidly under natural conditions because of the high heat capacity of water.

Gradual seasonal changes in temperature allow organisms to acclimatize to the harsher mid-seasonal winter or summer temperatures, but an abrupt temperature change can be lethal (Somero *et al*. 1996). With respect to temperature tolerance range, the high temperature limit is more variable than the low temperature limit (Freitas *et al.* 2010). Marbled crayfish are best cultured at temperatures of 18–25 °C, but can withstand temperatures below 8 °C and above 30 °C for many weeks, although mortality increases under such conditions and reproduction stops (Vogt *et al*. 2004). Seitz *et al*. (2005) showed that most marbled crayfish exposed to low temperatures (8 °C, 10 °C) survived these conditions: only two of eight individuals died at 8 °C, and the remaining six individuals survived for more than 40 days (i.e., the experimental period at 8 °C) and more than 100 days when they experienced temperatures of 10 °C to 8 °C, respectively. In the Paper **II** experiments, tanks experienced temperatures of less than 2 °C over 27 days, in addition to frequent temperature fluctuations in the 10 °C range, but marbled crayfish survived in both tanks. Winter water temperatures in natural bodies of water in Estonia are 0–2 °C, as confirmed by measurements from Piusa River, although the water temperature could be higher at greater depths, such as in lakes. It was not possible to keep the water temperatures as consistently low as those in Piusa River, so it is not possible to state definitivel that marbled crayfish can survive in Estonian climatic conditions, but this study showed that marbled crayfish are very resistant to extreme temperature conditions.

6.2.2. Effect of temperature and food on growth

Growth of crayfish has a genetic component, but is also greatly influenced by the rearing temperature and the food (Jones *et al*. 2000; Reynolds 2002). The effect of genetic component and rearing temperature on growth in these experiments can be excluded because all crayfish were genetically identical clone mates, and were reared under identical temperature conditions.

The growth pattern in crustaceans is a discontinuous process, with successive moults. Temperature is the most important environmental factor influencing crayfish growth, because it directly affects metabolic rate (Diaz *et al*. 2004). Higher temperatures shorten the moult cycle and lead to faster growth (Hartnoll 2001), but only within a suiTable range (García-Guerrero *et al*. 2003). Marbled crayfish increases in length and weight are strongly temperature dependent, being highest at 30 °C, and lowest at 15 °C. Seitz *et al*. (2005) noted that three marbled crayfish moulted at 10 °C. The current study indicated that marbled crayfish could moult at temperatures below 10 °C. At least two crayfish moults were recorded while the water temperature fluctuated between 5.1 °C to 9.5 °C, over 30 days in Tank 2 (Paper **II**).

Protein is the most expensive component in balanced feed and is probably the most important feed element for the growth of cultured species (Jones & Ruscoe 1996). The study in Paper **III** indicated that growth was significantly faster among crayfish fed with astaxanthin-rich feed than among crayfish fed with astaxanthin-free feed, although Harpaz *et al*. (1998) indicated that growth and survival of the crayfish were not affected by the addition of carotenoids to their diet. However, this can be explained by the higher protein content of the discus feed rather than its astaxanthin content.

6.2.3. Effect of temperature and food on reproduction

Temperature is one of the most important factors regulating egg development for several crustacean species (Choy 1991; Arshad *et al*. 2006). The effect of temperature on gonadal development and spawning of freshwater crayfish at different temperatures are different (Osalde *et al*. 2004). For example, in *P. clarkii*, embryonic development can last only 2–3 weeks (Vogt 2013), whereas in astacid crayfish, embryonic development can last 6–9 months in natural conditions (Reynolds *et al*. 1992).

Eleven marbled crayfish of different age and size (TL between 46–56 mm) were used in the study (Paper **II**). These crayfish lived in tanks at approximately 21 °C before their use in these experiments. During the experiments, water temperature decreased below 10 °C, all eggs of individuals were orange, and the animals moulted or died by the end of the experiment. The marbled crayfish is a warm water species, and the reproductive cycle stops at temperatures below 10 °C, which was confirmed by this

study. There are few studies of temperature limiting marbled crayfish reproduction, but *P. clarkii* can copulate and spawn in temperatures of 10 °C or lower (Cooper *et al*. 2011).

The native distribution of *P. fallax* covers the Florida Peninsula and southern Georgia in the United States of America (approximate 30.45°N latitude) (Hobbs 1981). The average minimum winter air temperatures fall below 6 °C (Myers & Ewel 1990) in the northern part of this range. In Estonia, marbled crayfish cannot reproduce all year round due to low water temperatures between autumn and spring, but during the summer, when average water temperatures are above 20 °C, they could reproduce at least once per year. Rennit (2011) showed that marbled crayfish started spawning when the water temperature reached 15.8 °C. Both of our winter experiments showed that the marbled crayfish that survived low temperatures spawned soon after the experiment, when the water temperature in their aquaria was increased to 20 °C. Eggs hatched normally and the progeny survived (Paper **II**).

An unbalanced or incomplete diet causes poor reproductive performance or may even stop reproduction (Izquierdo *et al*. 2001; Mengqing *et al*. 2004). During the reproduction and growth of crustaceans, limited carotenoids can lead to nutritional imbalances, physiological alterations and diseases (Harrison 1990; Scott 1999). "There have been few investigations about the carotenoids and retinoids the regulatory function and induction of maturation by nutritional agents such as carotenoids and retinoids, which in vertebrates are known to carry out important functions in protein expression and cellular differentiation" (Liňán-Cabello & Jesús 2004). Our study showed that astaxanthin and protein-rich feed did not accelerate marbled crayfish maturation. Marbled crayfish fed without astaxanthin in the feed became mature earlier than crayfish fed with astaxanthin-containing feed. However, by the end of the feeding trial, the proportions of crayfish with eggs was similar in both feeding groups (Paper **III**).

6.2.4. Effect of temperature and food on behaviour

Most crustaceans avoid harsh conditions by moving to an area that is more suiTable for the physiological functions that ensure survival, growth and reproduction (Gonzalez *et al*. 2010). Crayfish can avoid some temperatures when they have a choice (Hobbs 1981; Payette & McGaw

2003). *Astacus astacus* avoids warm temperatures (> 20 °C) in a 10–25 °C gradient and water colder than 10 °C in a cooler gradient (4–18 °C) (Kivivuori 1994). This implies that crayfish, and perhaps crustaceans generally, can detect temperature, either directly through thermal sensory reception or from changes in physiological conditions due to temperature changes (Chung *et al.* 2012).

The studies reported here showed that decreasing temperature (Paper **II)** and different feed (Paper **III**) changes marbled crayfish behaviour. Crayfish hid in shelters most of the time in both experiments in Paper **II**. When the water temperature decreased to less than 2 °C, crayfish came out of their shelters and lay motionless on the bottoms of the tanks. Crustaceans must rely on behavioural thermoregulation, on thermal acclimation capacity, and on adopting dormant or resting stages to survive temperature extremes (Lagerspetz & Vainio 2006). In our study, marbled crayfish became more active at temperatures above 6 °C and started eating at temperatures above 12 °C (Paper **II**).

It has been noticed that marbled crayfish have a more docile nature compared to other crayfish species, having mostly a conflict-avoidance and hiding strategy to threats (Lukhaup 2003). Our study indicated that mortality was higher due to the faster growth of marbled crayfish in the astaxanthin-rich feeding group due to cannibalism (Paper **III**).

6.2.5. Effect of food on colouration

Crustaceans have a wide variety of colour variations depending on species and genetic factors, moult stage, age, diet and environmental factors such as light regime and background colour (Aiken & Waddy 1987; Parisenti *et al*. 2011; You *et al*. 2006). In our study, all crayfish were genetically identical, were of the same age and were reared under the same conditions, excepting only the diet (Paper **III**). It is known that astaxanthin concentration is an appropriate indicator of body colour of shrimp (Nègre-Sadargues *et al*. 2000; Stepnowski *et al*. 2004), and it is the primary pigment which influences crustacean shell colour (Wade *et al*. 2005). It has been shown that astaxanthin appears as a red pigment, but when complexed with various proteins, the light absorbance shifts and cause crustaceans to range in colour from green, yellow, blue to brown (Lorenz 1998). Thus, despite the fact that astaxanthin is the chromophore prosthetic group of the different carotenoproteins, many colours

can be achieved (Nur-E-Bordan *et al*. 1995). The results of our feeding trial (Paper **III**) showed that an astaxanthin-rich feed could change the initial four carapace colour variants (light blue, blue, light brown, dark brown) to uniformly blue within two months from the start of feeding, and the blue colour darkened uniformly over time. The crayfish which received the astaxanthin-free feed had lighter and more variable colouration in shades of grey, and the blue colouration disappeared all together. These results are generally consistent with those of Harpaz *et al*. (1998) who showed that the Australian redclaw crayfish (*Cherax quadricarinatus*), receiving feeds fortified with carotenoids, exhibited better carapace colouration than those in the control group, which were fed a diet to which no carotenoids had been added. In addition, Tanaka (1978) has suggested that crustaceans should be fed diets supplemented with carotenoids to prevent fading of their colouration. As the marbled crayfish has become very popular among hobby aquarists (Vogt 2008, 2010; Faulkes 2010), it is important to understand how different feeds affect the carapace colouration in order to develop specific feeds to obtain the desired colour of crayfish.

Some studies have demonstrated a genetic basis for colour variation in several NA freshwater crayfish species (Black & Huner 1980), and it was found that the blue phenotype, which can be easily distinguished from the normal colour phenotype in newly released juvenile yabbies (*Cherax destructor*), was inherited as an autosomal recessive trait (Walker *et al.* 2000). In our experiment (Paper **III**), we used genetically identical marbled crayfish batch mates, which demonstrated variations in colour and growth even if they were reared under identical conditions. In addition, it has been shown that marbled crayfish demonstrate differences in lifespan, reproduction, morphometric traits, behaviour and fluctuating asymmetry of sense organs (Vogt *et al*. 2008). Vogt *et al*. (2007) indicated that, despite being raised in the same environment, individual genotypes can map to numerous phenotypes *via* developmental variation, thus generating variability among clone mates and individuality in a parthenogenetic species.

6.3. A set of polymorphic tetranucleotide repeat microsatellite markers for discriminating *A. astacus*, *A. leptodactylus* and their hybrids

Using the next-generation Illumina MiSeq System it was shown that it was possible to develop, quickly and efficiently, a set of 48 tetranucleotide repeat microsatellite markers from which 25 were polymorphic in *A. astacus,* and can be used in various population and conservation genetic studies of this highly valued crayfish species in Europe (Paper **IV**).

In addition, 21 out of 48 loci were also successfully cross-amplified in *A. leptodactylus.* Fourteen loci were polymorphic and these are the first microsatellite loci that can be used in population genetic studies of this widely distributed freshwater crayfish species (Paper **IV**). Furthermore, 13 loci (both monomorphic and polymorphic) possessed species-specific allele size range in *A. astacus* and *A. leptodactylus* and can be used for the detection of possible hybrids between these sister species, whose current distribution overlaps to a great extent (Paper **IV**). Hybrids between *A. astacus* and *A. leptodactylus* have been obtained under laboratory conditions (Furrer *et al.* 1999) and their occurrence in the wild has been suggested based on morphological studies (Maguire *et al.* 2013). However, identification of hybrids based on morphological traits is less reliable than by using bi-parentally inherited diagnostic genetic markers such as microsatellites. Again, the practical usefulness of these 13 microsatellite loci for the detection and confirmation of potential hybrids should be tested on larger sample sizes of both species. It is quite probable that the real allele size range for each species will be significantly larger, especially after sampling from a broader geographical area, and may partially overlap in *A. astacus* and *A. leptodactylus*.

7. CONCLUSIONS

1. Despite a ban on their introduction, signal crayfish have been recorded at four sites in Estonia since 2008, and have caused the extinction of noble crayfish populations, at least at two sites to date. The abundance of signal crayfish has fluctuated between years, increased in one population, is low in two localities, and the species has disappeared from one at present. The dispersed pattern of distribution and lack of connections between signal crayfish localities indicates that these populations are the result of illegal human-assisted introductions. This is confirmed by the fact that outbreaks of crayfish plague in native *A. astacus* populations are caused by different *A. astaci* genotype groups – A, B and E. Thus, the signal crayfish is confirmed as a potential threat to the Estonian native noble crayfish (Paper **I**).

2. Considering another dangerous alien species, especially the marbled crayfish, our studies have shown that it can survive at temperatures below 6 °C for six months, and tolerates temperatures below 2 °C for at least one week. They can moult at temperatures below 10 °C and reproduce after surviving in extreme environmental conditions. This suggests that the marbled crayfish might be able to survive through North European winters and reproduce in the following summer (Paper **II**).

3. It was shown, that different levels of astaxanthin and protein in feed have a significant effect on carapace colouration, growth rate and mortality of marbled crayfish. Crayfish fed with an astaxanthin-rich feed exhibited uniformly the same dark blue carapace colouration, while crayfish fed with ordinary carp feed exhibited significantly lighter and more variable colouration in shades of grey. Growth was significantly faster among marbled crayfish fed with an astaxanthin-rich feed than among crayfish fed with an astaxanthin-free feed. Cannibalism increased due to faster growth, and the mortality was higher in the astaxanthin-rich feeding group. The type of feed had no effect on the maturation of marbled crayfish. Due to good adaptility and reproduction in different environmental conditions the marbled crayfish is dangerous to the survival of noble crayfish populations (Paper **III**).

4. It was shown that using next-generation sequencing technology it is possible to develop, quickly and efficiently, a set of 48 tetranucleotide repeat microsatellite markers of which 25 were polymorphic in *A. astacus* and 14 were polymorphic in *A. leptodactylus*. Furthermore, 13 loci (both monomorphic and polymorphic) possessed species-specific allele size range in *A. astacus* and *A. leptodactylus* and can be used for the detection of possible hybrids between these sister species with largely overlapping distribution area (Paper **IV**).

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SUMMARY IN ESTONIAN

Invasiivsed vähi võõrliigid ja nende ohustav mõju jõevähi (*Astacus astacus* L.) asurkondadele Eestis

Jõevähk on hinnatud toidu- ja harrastuspüügiobjekt Põhja-Euroopas. Kunagised külluslikud jõevähivarud on nüüdseks kahanenud kõikjal Euroopas, seda peamiselt invasiivsete vähi võõrliikide leviku, vähikatku, elupaikade hävimise ja ülepüügi tõttu. Eestis on jõevähk ainus põline vähiliik, keda esineb rohkem kui 255-s looduslikus veekogus. Kõige rohkem on vähirikkaid veekogusid Saaremaal ja Lõuna-Eestis.

Invasiivsete vähi võõrliikide levik on üks peamisi Euroopa magevee vähivarude kahanemise põhjusi (Holdich *et al*. 2009). Põhja-Ameerika päritolu vähiliigid on agressiivsemad, viljakamad ja keskkonnatingimuste muutuste suhtes vastupidavamad kui kohalikud vähiliigid (Souty-Grosset *et al*. 2006). Võõrvähi liikide levikust tulenev peamine oht on vähikatk, mida põhjustav oomütseet *Aphanomyces astaci* on Euroopa vähiliikidele, sh jõevähile, enamasti surmav. Ameerikast pärit vähi võõrliigid ise on katku suhtes peaaegu immuunsed. Saaremaa oli kuni 2006. a katkupuhanguni oma rikkalike ja vähikatkust puutumata vähivarude poolest (Paaver & Hurt 2009) kogu Euroopas unikaalne koht. Lisaks vähikatkule avastati 2010. a Saaremaal, kus seni oli levinud vaid kodumaine jõevähk, Põhja-Ameerikast pärit vähi võõrliik – signaalvähk (*Pacifastacus leniusculus*).

Lisaks vähikatkule võib vähkide massilise suremuse põhjuseid olla ka teisi, kuid vähivarude kaitse ja majandamise seisukohast on vähkide hukkumise puhul oluline tuvastada vähikatku olemasolu, et rakendada meetmeid katku edasise leviku tõkestamiseks. Vähikatku ja selle tüvesid saab kindlaks teha molekulaargeneetiliste meetoditega (Vrålstad *et al.* 2009; Grandjean *et al.* 2014).

Kõige levinum invasiivne vähi võõrliik Euroopas on signaalvähk (Kouba *et al*. 2014). Kuni 2008. aastani oli Eesti Iirimaa, Norra, Horvaatia, Slovakkia ja Venemaa kõrval üks viimaseid riike Euroopas, kus vähi võõrliigid polnud teadaolevalt levinud (Kouba *et al*. 2014). Praegu esineb signaalvähk nii Eestis kui ka teistes nimetatud riikides, v.a Iirimaal.

Potentsiaalne oht Euroopa kohalike vähiliikide jaoks on viimaste kümnendite jooksul hoogustunud dekoratiivsete vähi võõrliikide müük akvaariumikaubanduses, mis on invasiivsete vähi võõrliikide puhul muutunud peamiseks levikuteeks Euroopasse (Chucholl *et al*. 2012). Üheks enim kaubeldavaks vähiliigiks on marmorvähk (*Procambarus fallax* f. *virginalis*), kes avastati esmakordselt Saksamaa ja Austria akvaariumikaubanduses 1990ndate keskel (Lukhaup 2001; Scholtz *et al*. 2003; Martin *et al*. 2010) ning on populaarne ka Eesti akvaristide seas. Kesk-Euroopas on marmorvähk levinud juba ka looduses ja tema asurkonnad suurenevad järjest partenogeneetilise paljunemisviisi tõttu. See seab ohtu Euroopa kohalikud vähiliigid (Souty-Grosset *et al*. 2006; Chucholl & Daudey 2008; Chucholl 2011). Marmorvähi potentsiaalse ohu hindamiseks jõevähile Eesti looduses tuleb hinnata tema ellujäämust talvistes tingimustes ja teisi bioloogilisi omadusi nagu kasv ja paljunemine.

Kohalike vähiliikide asurkondade elujõulisuse ja keskkonna-muutustega kohanemise tagamisel on võtmetähtsusega liigisisese geneetilise mitmekesisuse säilitamine ja kaitse. Selle uurimiseks kasutatakse geneetilisi markereid, näiteks mikrosatelliite, mida läheb vaja morfoloogiliselt sarnaste liikide ja nende hübriidide (näiteks jõevähi ja kitsasõralise vähi hübriidid) usaldusväärseks eristamiseks. Esimesed jõevähi mikrosatelliitmarkerid (kokku 19) töötasid välja Kõiv *et al*. (2008, 2009). Kuna kitsasõralise vähi jaoks liigispetsiifilised mikrosatelliitmarkerid seni puudusid, tuli välja töötada mikrosatelliitmarkerid, mis oleksid kasutatavad nii jõevähi kui ka kitsasõralise vähi puhul ning võimaldaksid tuvastada kahe liigi võimalikke hübriide.

Jõevähile potentsiaalselt ohtlike invasiivsete vähi võõrliikide mõju hindamiseks seati käesolevas väitekirjas järgmised eesmärgid ja hüpoteesid:

1) saada ülevaade signaalvähi seisundist ja levikust Eestis ning tema potentsiaalsest rollist hiljutistes vähikatku puhangutes jõevähi asurkondades **(I)**;

   Hüpotees: *Hiljutised vähikatku puhangud jõevähi asurkondades Eestis on vähemalt osaliselt seotud invasiivsete vähi võõrliikide levikuga.*

2) hinnata veetemperatuuri ja erinevate söötade mõju marmorvähi ellujäämusele, kasvule, paljunemisele, käitumisele ja kesta värvusele (**II, III**);

Hüpotees: *Marmorvähk on jõevähile potentsiaalselt ohtlik oma kasvu- ja paljunemiskiiruse tõttu erinevates keskkonnatingimustes ning on võimeline üle elama Eesti talviseid tingimusi.*

3) töötada välja uued polümorfsed tetranukleotiidsed mikrosatelliitmarkerid jõevähi ja potentsiaalselt ohtliku vähi võõrliigi – kitsasõralise vähi ning nende võimalike hübriidide geneetiliseks tuvastamiseks **(IV)**;

   Hüpotees: *Kasutades järgmise põlvkonna DNA nukleotiidse järjestuse määramise tehnoloogiaid, on võimalik välja töötada polümorfsed mikrosatelliitmarkerid jõevähile, mida saab osaliselt kasutada ka kitsasõralise vähi puhul ning kahe liigi võimalike hübriidide tuvastamisel.*

Pärast signaalvähkide avastamist Eesti neljas veekogus aastatel 2008 (Mustjõgi), 2010 (Riksu oja), 2012 (Vääna jõgi) ja 2016 (Pärnu jõgi), teostati nimetatud asurkondades vähivarude seisundi ja edasise leviku hindamiseks iga-aastased katsepüügid. Info jõevähi esinemise kohta neis veekogudes juba enne signaalvähkide leidu saadi EMÜ katsepüükide andmebaasist, mis sisaldab vastavaid andmeid alates 1989. aastast.

Vähikatku uurimiseks koguti andmeid kokku 16-st veekogust, kus on katkulaadne suremus fikseeritud alates 2006. aastast. Vähikatku molekulaardiagnostiline analüüs teostati aastatel 2006–2016 vastavalt Vrålstad *et al*. (2009) qPCR metoodikale Norra veterinaarinstituudis (2007), Eesti maaülikoolis (2010–2011) ja Poitiersi ülikoolis (2014–2016), aastatel 2009–2010 vastavalt Oidtmann *et al*. (2004) metoodikale Soome toiduohutusameti laboris. Vähikatku tüvede määramisel kasutati Grandjean *et al*. (2014) metoodikat ja see tehti Poitiersi ülikoolis Prantsusmaal (2014, 2016).

Marmorvähi ellujäämust Eesti talvistes kliimaoludes hinnati 2011/12 aasta talvel välitingimustes EMÜ vesiviljeluse õppetoolis korraldatud basseinikatse põhjal. Katse viidi läbi 110 päeva jooksul kahes ühe m^3 suuruses basseinis, kus osales 50 marmorvähki. Vähkide suremuse ja veetemperatuuri vahelist seost hinnati katseaja kuude keskmiste veetemperatuuride ± SE ning ellujäänud vähkide arvu kaudu.

Erinevate söötade mõju marmorvähi ellujäämuse, kasvu, paljunemise, käitumise ja kesta värvuse hindamiseks korraldati 2013. aastal akvaariumikatse 90 marmorvähiga. 123 päeva jooksul võrreldi kahe erineva

astaksantiini- ja proteiinisisaldusega sööta (ketasahvena ehk diskuse ja karpkala sööt) kolmes korduses, kokku kuues 55-liitrises akvaariumis. Värvust mõõdeti kaheksas värvitoonis. Kõik katses osalenud vähid mõõdeti ja kaaluti ning nende värvust hinnati nii katse alguses, keskel kui ka lõpus. Katse jooksul registreeriti surnud vähkide arv ning suguküpsuse saabumine.

Uute polümorfsete tetranukleotiidsete mikrosatelliitmarkerite väljatöötamiseks jõevähile ja kitsasõralisele vähile kasutati uue põlvkonna sekveneerimistehnoloogiat Illumina MiSeq platvormil ja tarkvara QDD v. 3.1 (Meglécz *et al*. 2014). 48 praimeripaari valiti vastavalt Meglécz *et al*. (2014) kriteeriumitele ning nende amplifitseerimist ja polümorfismi testiti kaheksal jõevähil ja kaheksal kitsasõralisel vähil. Selleks kasutati mikrosatelliidilookuste genotüpiseerimiseks Applied Biosystems 3500 DNA analüsaatorit (Life Technologies, USA).

Signaalvähi invasiivsete asurkondade uuringud Eesti neljas veekogus näitasid, et signaalvähk on jõevähi välja tõrjunud vähemalt kahest elupaigast. Samas on signaalvähi arvukus aastatega kõikunud ning on järjest suurenenud vaid ühes veekogus. Ühest veekogust on signaalvähk, aga ka jõevähk, hoopiski kadunud. Arvukus on madal kahes veekogus, ühes neist esineb signaalvähk koos jõevähiga. Signaalvähi leiukohtade paiknemise põhjal võib arvata, et ta on Eestisse illegaalselt sisse toodud, kuna neil veekogudel puuduvad ühendused naaberriikide veekogudega. Vähikatku puhangud jõevähi asurkondades on põhjustanud nii *A. astaci* A, B kui E genotüübigrupid, mis näitab, et katku levitab signaalvähk, välistada ei saa ka teiste võõrliikide mõju.

Kodumaisele jõevähile potentsiaalselt ohtliku marmorvähi uuringud näitasid, et nimetatud liik on suuteline ellu jääma alla 6 °C vees vähemalt kuus kuud ning talub alla 2 °C temperatuuri vähemalt nädala. Marmorvähk suudab kasvada alla 10 °C vees ja paljuneda pärast ekstreemsete keskkonnatingimuste üleelamist. Sellest tulenevalt võib järeldada, et marmorvähk suudab ellu jääda ka Põhja-Euroopa, sh Eesti talvistes kliimatingimustes ning anda suvel järglasi. Vältida tuleb tema sattumist akvaariumidest loodusse.

Erineva astaksantiini- ja proteiinisisaldusega söödad mõjutasid marmorvähkide kooriku värvust, kasvu, kasvukiirust ja suremust. Astaksantiini- ja proteiinirikkamal söödal olnud marmorvähkide värvus muutus ühtla-

selt tumesiniseks, samal ajal astaksantiinivaba ja proteiinivaesemat sööta saanud vähkide värvus varieerus helesinise ja helepruuni ning heleda ja tumeda halli värvuse vahel. Astaksantiini- ja proteiinirikkamal söödal olnud marmorvähkide kasv oli karpkala sööta saanud vähkidest oluliselt kiirem. Kiirem kasv soosis aga kannibalismi ja suuremat suremust. Suguküpsuse saabumises olulist erinevust mõlema söödagrupi vähkide seas ei täheldatud. Hea kohanemis- ja paljunemisvõime tõttu erinevates keskkonnatingimustes ning lisaks vähikatku võimalikule levitamisele on marmorvähk jõevähile potentsiaalselt ohtlik.

Järgmise põlvkonna DNA sekveneerimistehnoloogiate kasutamine võimaldas kiiresti ja tõhusalt välja töötada 48 tetranukleotiidsete kordustega mikrosatelliitmarkerit, millest 25 olid polümorfsed jõevähil ja 14 kitsasõralisel vähil. Lisaks olid 13 lookusel jõevähile ja kitsasõralise vähile spetsiifilised alleelide suurusvahemikud, mida saab kasutada nende kahe liigi hübriidide usaldusväärseks tuvastamiseks piirkondades, kus nende liikide leviala kattub.

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I

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INVASION NOTE

First records of the non-indigenous signal crayfish (*Pacifastacus leniusculus*) and its threat to noble crayfish (*Astacus astacus*) populations in Estonia

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Abstract This study gives an overview of status and distribution of signal crayfish (*Pacifastacus leniusculus*), the first NICS in Estonia and its influence on native noble crayfish (*Astacus astacus*) populations. The first specimen of signal crayfish was caught during the monitoring of noble crayfish in North Estonia in 2008. The signal crayfish has since been found in three additional sites. Test fishing has indicated that the abundance of signal crayfish has been fluctuating between years and among localities. It has had strong negative impact on abundance of one noble crayfish population. The disconnected distribution of signal crayfish strongly suggests that these populations are the result of human-assisted introductions. Real-time PCR analyses proved that signal crayfish carry the causative agent of the crayfish plague, an oomycete *Aphanomyces astaci*, thus contributing to its spread. Mortalities in noble crayfish populations had been caused by *A. astaci* strains from A, B and E genotype group.

Keywords Invasive crayfish · Native crayfish · *Aphanomyces astaci* · Disease outbreak · Genotyping

Introduction

The introduction of non-indigenous crayfish species (NICS) is one of the major causes of extinction of indigenous crayfish species (ICS) in European freshwaters (Holdich et al. 2009). Progressively spreading NICS often exhibit devastating effects on ICS stocks as well as on entire ecosystems across European countries (Rodríguez et al. 2005).

The North American (NA) signal crayfish (*Pacifastacus leniusculus*) is the most widespread NICS in Europe, being first introduced to Sweden in 1959. By 2014, it occured in at least 29 European regions (Kouba et al. 2014). Crayfish species of NA origin are latent carriers of the crayfish plague (Alderman et al. 1990) caused by the oomycete *Aphanomyces astaci* (Unestam 1972). Crayfish plague is lethal in most cases to all crayfish species not originating from NA (OIE 2012).

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The noble crayfish (*Astacus astacus*) is the only ICS in Estonia and occurs in more than 255 sites (lakes and river stretches), but most sites present low densities, except for some populations in South-Eastern Estonia and on the island of Saaremaa (Paaver and Hurt 2009). The main factors causing the decline of Estonian crayfish populations since early 1900s are crayfish plague, habitat deterioration, mink (*Mustela vison*) and eel (*Anguilla anguilla*) predation and fishing (Tuusti et al. 1993).

Until 2008 Estonia was one of the last countries in Europe where NICS were not recorded. To protect native noble crayfish, the Estonian Nature Conservation Act prohibits introduction of non-native species into the wild, bringing live specimens of the signal crayfish, narrow-clawed crayfish (*Astacus leptodactylus*) and spiny-cheek crayfish (*Orconectes limosus*) into Estonia, or conducting transactions with live specimens of these species.

In 2016, there were 23 noble crayfish farms having operating licences from the Veterinary and Food Board in Estonia. However, crayfish plague outbreaks, together with information about efficient and cheap exploitation of signal crayfish populations in Finland, increased the interest of crayfish farmers and owners of water bodies in introducing crayfish plague resistant crayfish species. Illegal introductions of crayfish plague-carrying NICS create potential dangers to native noble crayfish populations and crayfish farms, because they are already spread in water bodies of neighbouring countries such as Sweden, Finland and Latvia (Kouba et al. 2014).

The aim of this study was to get an overview of the status and distribution of signal crayfish and its involvement in a series of plague outbreaks taken place recently in noble crayfish populations in Estonia.

Detection and distribution of signal crayfish in Estonia

Signal crayfish have now been found in four sites in Estonia (Supplementary Table 1; Fig. 1). The first three signal crayfish populations were found during monitoring of noble crayfish carried out by the Department of Aquaculture of Estonian University of Life Sciences (EULS) by means of cylindrical Mjärde Lini traps. The fourth signal crayfish population was found by local fishermen. After the discovery of signal crayfish, monitoring was conducted at all these sites yearly (Supplementary Table 1). For every trapping session, catch per unit effort (CPUE; the number of caught crayfish per trap night) was recorded at each site (Supplementary Table 1). Information about the occurrence of noble crayfish in these rivers before the detection of signal crayfish was obtained from a database of standardized test fishings and crayfish stockings of the Department of Aquaculture of EULS which was held since 2003 and includes data back to 1989.

The first specimen of signal crayfish was caught in Mustjõgi River, Harju County in 2008 (Fig. 1). This river is 38 km long with a catchment area of 98.8 km^2. The noble crayfish population had become extinct there but recovered after restockings in 1997–1999. Test fishings in 2005 showed the presence of noble crayfish (CPUE 1.2), but after the detection of signal crayfish in 2008, noble crayfish were not found except in 2014 (a single specimen). No signal or noble crayfish were found in extensive test fishings in Mustjõgi River and its tributaries in 2009 (Supplementary Table 1). In 2010–2012 few signal crayfish were found at the same site, in a 25–30 m long section of the river (Supplementary Table 1). During the years 2013–2016 no signal crayfish were caught in test fishings.

The second signal crayfish population was found in 2010 on the island of Saaremaa in Riksu Stream, which is 19.6 km long with a catchment area of 49.4 km^2. Additional test fishing with an increased number of trap nights was carried out and 61 (CPUE 0.12) signal crayfish were caught from a 500 m section downstream of the previous site in Riksu Stream (Supplementary Table 1). Noble crayfish were not found at that site although in the early 2000s there was a noble crayfish population. About 50 noble crayfish were caught per 10 m in 2002 (unpublished data of EULS). In 2011 and 2012 a new site was found just upstream but the number of signal crayfish in the total catch was lower despite an increase in the number of trap nights (Supplementary Table 1). In 2013, new signal crayfish sites were found further upstream, and up to 2014 signal crayfish occurred in about three km section of Riksu Stream. The total catch in 2013–2016 had increased (CPUE 0.19 up to 3.31) compared to the first 3 years (Supplementary Table 1). The estimated migration rate of signal crayfish in Riksu Stream during the last 2 years has been one km upstream.

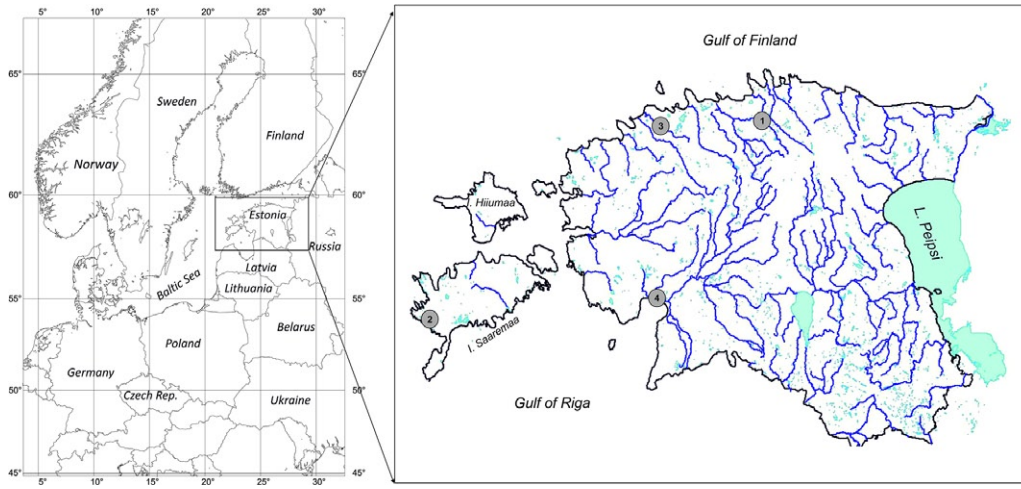

Fig. 1 The signal crayfish locations in Estonia and the year of first reporting: *1* Mustjõgi River, Harju County (2008); *2* Riksu Stream, island of Saaremaa (2010); *3* Vääna River, Harju County (2012); *4* Pärnu River, Pärnu County (2016)

The third signal crayfish population was found in 2012 in Vääna River, Harju County. It is 64.3 km long with a catchment area of 315 km^2. In the end of the 1990s there was a dense noble crayfish population (CPUE 4.8). The situation in Vääna River was different from other rivers because both noble crayfish and signal crayfish are living in sympatry. In 2012 one noble crayfish and one signal crayfish were found in 135 trap nights (Supplementary Table 1). In 2013, test fishing included more traps (300 trap nights) and one signal crayfish was found. At the same time, noble crayfish dominated in catch. In 2014, abundance of both species had increased. In 2015 and 2016 fewer signal and noble crayfish were caught using the same number of traps (Supplementary Table 1).

The fourth signal crayfish population was found in 2016 by local fishermen in Pärnu Bay, 2 km from the coast and in the mouth of Pärnu River in the middle of Pärnu City (Pärnu County). Pärnu River is 144.5 km long with a catchment area of 6836.5 km^2. In a following test fishing 16 signal crayfish and one noble crayfish were caught from the river during 100 trap nights (Supplementary Table 1; Fig. 1).

Detection of crayfish plague

To get an overview of crayfish plague occurrence in Estonia samples from 16 populations where mortalities had occurred or from cage experiments were analysed for *A. astaci* (Supplementary Table 2). Molecular tests for crayfish plague detection were conducted in four different laboratories based on Oidtmann et al. (2004) and Vrålstad et al. (2009) methodology (Supplementary Table 2). *Aphanomyces astaci* multilocus genotype (SSR) was determined according to Grandjean et al. (2014) methodology (Supplementary Table 2).

Multilocus genotype group was determined in case of four crayfish mass mortalities. A sample from Laugi Stream (2007) on the island of Saaremaa showed *A. astaci* multilocus genotype SSR-E and a sample from Pärlijõgi River (2010) in the Võru County showed *A. astaci* multilocus genotype SSR-B. Other two cases in Härjanurme fish and crayfish farm (2010) in Jõgeva County and Avijõgi River (2015) in the East-Viru County exhibited *A. astaci* multilocus genotype SSR-A (Supplementary Table 2). Crayfish plague analyses in signal crayfish watercourses—Mustjõgi River, Riksu Stream and Vääna River indicated presence of *A. astaci* (Supplementary Table 2) but genotype could not be determined.

Discussion

The spread of NICS is a combination of natural expansion and human-assisted introductions which

may be both deliberate and accidental (Peay and Füreder 2011). In our case, natural migration is not an issue since the discovered locations are not connected to the waters inhabited by the species in neighbouring countries. Also, there is no direct connection between the signal crayfish-inhabited rivers in Estonia. The main vector of introduction of NICS in Estonia may be the international trade with alive crayfish and the interest of increasing number of crayfish farmers (Paaver and Hurt 2009) in the introduction of alien species. The threat to the noble crayfish from illegal introduction, catching, trade and farming of NICS (Paaver and Hurt 2009) set a need for the improved legislation restricting the spread of NICS. These changes were included into the Nature Conservation Act and adopted in 2004.

Our study showed that the crayfish plague was detected at first time on the island of Saaremaa in 2006, which was 4 years earlier than found signal crayfish. Crayfish plague caused collapse of the stocks in the three crayfish farms during 2006–2007 and in two wild populations, in rivers that were connected to the Pähkla fish and crayfish farm on the island of Saaremaa (Supplementary Table 2). Analysis of one sample from 2007 Laugi Stream outbreak showed *A. astaci* multilocus genotype SSR-E which is originally isolated from spiny-cheek crayfish (*Orconectes limosus*) (Kozubíková et al. 2011). Based on these data we might assume that at least the occurrence of crayfish plague genotype group E on the island of Saaremaa could be linked to the trade with alive crayfish with Lithuania. No live *O. limosus* specimens have been found in Estonia so far but its occurrence is confirmed in Latvia (Briede 2011) and Lithuania (Arbăciauskas et al. 2011). *Aphanomyces astaci* could spread to the island of Saaremaa also by the careless transfer of contaminated crayfish traps or alive fish transport. The presence of genotype group A involved in Härjanurme fish and crayfish farm (2010) and Avijõgi River (2015) mass mortalities and genotype group B (strain originating from *P. leniusculus*) in Pärlijõgi River (2010) was not really surprising. Recently, Vrålstad et al. (2014) and Maguire et al. (2016) reported that both genotype groups were responsible of a large series of outbreak in *A. astacus* stock in Norway and Croatia, respectively. The same explanation can be given to the outbreak in Pärlijõgi River where was moderate density of noble crayfish population (CPUE 3.0) in the middle of 2000s. In 2010 detected crayfish plague outbreak and *A. astaci* genotype group analysis revealed B strain (Supplementary Table 2). Signal crayfish has never been detected in this river. Pärlijõgi River belongs to Gauja River watershed in Estonia and Latvia, but by data of Briede (2011), signal crayfish does not habit there. However, we cannot exclude other animals (e.g. semiaquatic mammals or birds), which can also spread the spores (Makkonen et al. 2013).

There is no information on how signal crayfish spread to Estonia, but this may already have happened before 2008. No evidence about legal introductions of NICS to Estonia has been found. Strict regulation of import of live crayfish to certain European countries (Peay 2009) and intensive proactive conservation measures to protect the ICS (Holdich et al. 2009) have not been sufficient to reduce the trade and spread of NICS (Peay 2009). Trade in ornamental freshwater crayfish has grown rapidly in the last decade and many aquarium shops in Estonia have sold marbled crayfish that are able to survive in North European countries and transmit the crayfish plague (Keller et al. 2014; Kaldre et al. 2015; Mrugala et al. 2015).

Our data indicate different patterns of development of introduced NICS populations. So far, signal crayfish have been responsible for disappearance of the noble crayfish population in Estonia at least in two sites—Mustjõgi River and Riksu Stream. In the Mustjõgi River noble crayfish have not been found after the discovery of signal crayfish, but today, the signal crayfish population seems to have been lost as well. The last signal crayfish in Mustjõgi River was seen in 2012. During 2015 and 2016, 1 000 noble crayfish in total have been restocked to the Mustjõgi River and have been survived so far. Five noble crayfish (CPUE 0.1) were caught in 2016 from the same place where the signal crayfish was detected. Signal crayfish were probably brought to the island of Saaremaa in 2004–2005 and have been responsible for the disappearance the noble crayfish population in the Riksu Stream in the beginning of the 2000s. First detected in 2010, during the last 6 years the signal crayfish population has been growing and there are no obstacles to spread in both directions—either downstream (via Riksu Lake towards the sea) or upstream. Until 2013, there were attempts to dry part of the river inhabited by signal crayfish by constructing a new side channel. However, new sites with signal crayfish occurred upstream, making this activity senseless and

turning the possible eradication almost impossible. The case of Pärnu bay also shows that signal crayfish can tolerate brackish (salinity 2–3 ppt) conditions and spread via coastal areas.

Noble crayfish populations did not disappear from all the signal crayfish locations. Although signal crayfish are chronic carriers of *A. astaci* and are also capable of occupying habitats of European ICS, having wider environmental tolerance (Peay and Füreder 2011), in Estonia noble crayfish still persist in sympatry with signal crayfish in two localities—in Vääna and Pärnu Rivers. Signal crayfish were found together with noble crayfish in a small (200 m) site in Vääna River in 2012 and test fishing in each year showed the presence of both species at the same site (Supplementary Table 1). Crayfish plague analyses of signal crayfish showed a low level of *A. astaci*, but noble crayfish were not infected (Supplementary Table 2). The number of analysed specimens was small and period of our study short, thus monitoring including more individuals and longer time should be carried out. Still our study confirmed that permanent coexistence between ICS and NICS is possible as also described in other studies (Schrimpf et al. 2013; James et al. 2017).

Conclusions

Despite a ban on introduction, signal crayfish have been recorded at four Estonian sites since 2008 and have caused the disappearance of noble crayfish populations at least in two sites so far. Abundance of signal crayfish has been fluctuating between years, increased in one population, is low in two localities and disappeared from one place. The pattern of distribution and the fact that the water bodies with signal crayfish localities are not connected, strongly suggest that these populations are the result of illegal human-assisted introductions. It is confirmed by the fact that many outbreaks of crayfish plague in native *A. astacus* populations are caused by different *A. astaci* genotype groups—A, B and E.

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Supplementary Table 1. The number of trap nights, caught individuals of signal crayfish *Pacifastacus leniusculus* (*P.l.*) and noble crayfish *Astacus astacus* (*A.a.*) and total CPUE of test fishings during 2008–2016

Watercourse / coordinates	**2008**	**2009**	**2010**	**2011**	**2012**	**2013**	**2014**	**2015**	**2016**
Mustjõgi River (Harju county) 25° 36'48" E[1] 59° 17'50" N[1]									
No of traps	40	80	40	340	200	310	100	80	40
No of *P.l.*	1	0	5	7	1	0	0	0	0
No of *A.a.*	0	0	0	0	0	0	1	0	5
CPUE	0.03	0	0.13	0.02	0.01	0	0.01	0	0.13
Riksu Stream (Island of Saaremaa) 22° 05'13" E[2] 58° 11'39" N[2]									
No of traps	–	–	505	1,400	440	734	238	514	310
No of *P.l.*	–	–	61	50	31	140	256	743	1,027
No of *A.a.*	–	–	0	0	0	0	0	0	0
CPUE		–	0.12	0.04	0.07	0.19	0.81	1.45	3.31
Vääna River (Harju county) 24° 39'26" E[1] 59° 18'59" N[1]									
No of traps	–	–	–	–	135	300	160	180	180
No of *P.l.*	–	–	–	–	1	1	11	4	2
No of *A.a.*	–	–	–	–	1	18	22	5	1
CPUE	–	–	–	–	0.01	0.06	0.21	0.05	0.02
Pärnu River (Pärnu county) 24° 29'30" E[3] 58° 23'12" N[3]									
No of traps	–	–	–	–	–	–	–	–	100
No of *P.l.*	–	–	–	–	–	–	–	–	16
No of *A.a.*	–	–	–	–	–	–	–	–	1
CPUE	–	–	–	–	–	–	–	–	0.17

[1]given coordinates are midpoint of the trap line
[2]signal crayfish distribution area is 3 km upstream from the given coordinates
[3]after additional test fishing signal crayfish were found in two other locations in Pärnu River (24° 28'53" E, 52° 23'18" N and 24° 30'02" E, 58° 23'18" N).

Supplementary Table 2. Crayfish plague events in Estonia during 2006–2015

Sample origin[a]	Crayfish species[b]	Sampling year	No. and status of analysed individuals[c]	PCR/ qPCR result[d]	Highest agent level[e]	Geno-type group[f]	Analysis site[g]	Analy-sis year
Pihtla crayfish farm, Island Saaremaa, OB in 2006	*A.a*	2006	2 alive*	2 posit	A3	NT	NVI	2007
		2006	2 alive	2 posit	A2	NT	Univ-Poitiers	2016
Pähkla fish and crayfish farm, Island Saaremaa, OB in 2007	*A.a.*	2007	1 dead*	1 posit	A7	NT	NVI	2007
Põduste River, Island Saaremaa, OB in 2007, CE in 2008	*A.a.*	2007	11 dead*	8 posit	A7	NT	NVI	2007
		2008	1 alive	1 posit	A1	NT	Univ-Poitiers	2016
Laugi Stream, Island Saaremaa, OB in 2007	*A.a.*	2007	4 alive*	2 posit	A6	NT	NVI	2007
		2007	1 dead	1 posit	A3*	E	Univ-Poitiers	2016
Sõrve crayfish farm, Island Saaremaa, OB in 2007	*A.a.*	2007	2 alive*	1 posit	A2	NT	NVI	2007
Riksu Stream, Island Saaremaa	*P.l.*	2011	4 alive	3 posit	NK	NT	EULS	2011
		2011	2 alive**	2 posit	A3	NT	Univ-Poitiers	2014
		2013	5 alive	1 posit	A2	NT	Univ-Poitiers	2014
		2015	6 alive	4 posit	A2	NT	Univ-Poitiers	2015
Masa River, Island Saaremaa, OB in 2013	*A.a.*	2013	4 dead	1 posit	A2	NT	Univ-Poitiers	2014
		2013	1 alive	1 posit	A2	NT	Univ-Poitiers	2016
Leevi River, Põlva, OB in 2007, CE in 2009	*A.a.*	2007	2 dead*	2 posit	A5	NT	NVI	2007
		2007	2 dead	2 neg	A0	NT	Univ-Poitiers	2016
		2009	1 died*, 1 alive*	2 posit*	NK	NT	EVIRA	2009
Mustjõgi River, Võru, DD in 2008	*A.a.*	2008	1 alive*	1 posit*	NK	NT	EVIRA	2009
Mustjõgi River, Harju, CE in 2009	*A.a.*	2009	2 dead*	2 posit*	NK	NT	EVIRA	2009
	P.l.	2009	1 alive*	1 neg*	NK	NT	EVIRA	2009
Lake Selja, West-Viru, OB in 2009	*A.a.*	2009	3 dead	3 posit*	NK	NT	EVIRA	2009
		2009	2 dead	2 posit	A1	NT	Univ-Poitiers	2016
Pärlijõgi River, Võru, OB in 2008-2010	*A.a.*	2010	4 dead	3 posit*	NK	NT	EVIRA	2010
		2010	1 dead	1 posit	NK	NT	EULS	2010
		2010	1 dead	1 posit	A4*	B	Univ-Poitiers	2014
		2010	2 dead	2 posit	A5*	B	Univ-Poitiers	2016
Härjanurme fish and crayfish farm, Jõgeva, OB in 2010	*A.a.*	2010	1 dead	1 posit	NK	NT	EULS	2010
		2010	1 dead	1 posit	A4*	A	Univ-Poitiers	2016
Taebla River, West, DD in 2010	*A.a.*	2011	1 alive	1 posit	NK	NT	EULS	2011
		2011	1 alive**	1 posit	A3	NT	Univ-Poitiers	2014
Vääna River, Harju	*P.l.*	2015	4 alive	2 posit	A1	NT	Univ-Poitiers	2015
	A.a.	2015	2 alive	2 neg	A0	NT	Univ-Poitiers	2015
Avijõgi River, East-Viru, OB in 2015	*A.a.*	2015	3 dead	3 posit	A4*	A	Univ-Poitiers	2015

[a] Sample origin: All samples originate from suspected incidences of crayfish plague in Estonia. The sample origin listed includes the name of population (farm, lake, river or stream) and county. The crayfish samples originate either outbreak (OB), decline of density (DD) or cage experiment (CE) that has been used for crayfish plague surveillance in the years after an outbreak.

[b] *A.a. = Astacus astacus; P.l. = Pacifastacus leniusculus.*

[c] *Crayfish tissue material or DNA-isolates were not stored, ** DNA used in analyses was extracted at EULS in 2011.

[d] *Analyses at the Finnish Food Safety Authority (EVIRA) based on the Oidtmann *et al.* (2004) PCR methodology. Others based on the Vrålstad *et al.* (2009) qPCR methodology.

[e] Only the highest detected agent level is given. Results marked * have been confirmed by sequencing. NK = not known – the agent level was not included in the protocol.

[f] Genotype group determined based on microsatellite markers. NT = not tested.

[g] NVI = Norwegian Veterinary Institute; EVIRA; Univ-Poitiers = University of Poitiers; EULS.

II

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4

A Preliminary Study on the Tolerance of Marble Crayfish *Procambarus fallax* f. *virginalis* to Low Temperature in Nordic Climate

Katrin Kaldre,[1,a,*] Anton Meženin,[2] Tiit Paaver[1,b] and Tadashi Kawai[3]

Introduction

The trade of live ornamental freshwater crayfish has grown rapidly in the last decade and has become the major pathway for new Non-Indigenous Crayfish Species (NICS) introductions into Europe (Chucholl et al. 2012). The parthenogenetic marbled crayfish or Marmorkrebs (*Procambarus fallax* f. *virginalis*) (Scholtz et al. 2003, Martin et al 2010) had circulated in the European pet trade in laboratories since the 1990s (Vog 2008, 2010, Faulkes 2010), and several years ago, the first living samples were captured from natural European habitat (Soes and van Eekelen 2006). Since then, their known range is rapidly spreading in Central European countries, creating a new threat for European indigenous water ecosystems (Souty-Grosset et al. 2006, Chucholl and Daudey 2008, Chucholl 2011). People in Nordic European countries (especially Estonia) are concerned about the potential invasion of marble crayfish, because the Estonian Nature

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Conservation Act is intended to prevent the introduction and spread of non-native crayfish species in the wild. The law specifically lists signal crayfish, *Pacifastacus leniusculus*, narrow-clawed crayfish, *Astacus leptodactylus*, and spiny cheek crayfish *Orconctes limosus*, but marble crayfish are not yet listed. Unfortunately, in recent years, many aquarium shops in Estonia have sold marble crayfish for aquarists as pet species, and there are many websites selling marble crayfish. In a worst case scenario, the illegal release of a single marble crayfish from a hobbyist's home aquarium could establish a new population in Estonia, which then diffuses to all Nordic European countries!

Seitz et al. (2005) showed that marble crayfish could survive at low temperatures, 8°C, which is similar to groundwater temperature in Central Europe. Temperatures in Nordic countries' water bodies are much lower during winter, but there is no information about whether marble crayfish can survive temperatures as low as those experienced in Nordic countries, which is relevant to whether the marble crayfish can invade northern Europe. In this chapter we discuss the low temperature tolerance of marble crayfish as a factor in the potential invasion of marble crayfish in Nordic European countries.

Material and Methods

In order to examine low temperature water tolerance for marble crayfish and the possibility of invasion of marble crayfish into northern Europe, the survival, growth, behavior and reproduction of marble crayfish in low temperature were examined in the winter periods, from September 9, 2011 to April 18, 2012 (110 days) in an outdoor tank, in Estonia. In general, low temperature water tolerance depends on body size, so survival of two body size groups of marble crayfish was compared.

The marble crayfish individuals were bought from an Estonian aquarium shop and stocked into the aquarium at the Department of Aquaculture of the Estonian University of Life Sciences. They were reared in two 1 m^3 outdoor tanks. Twenty five larger animals (mean total length (TL) (mm) = 42.2 ± 6.4 SD, mean weight = 2.1 ± 0.4 SD (g)) were stocked into Tank 1, and 25 smaller animals (mean TL (mm) 31.6 ± 2.8 SD, mean weight 0.9 ± 0.2 SD (g)) were stocked into Tank 2. At the start and end of the experiment, the Total Length (TL), weight and number of eggs of each individual were measured. Dead animals or molting individuals were counted daily during the experiment. To make the water temperature in the experimental tanks similar to natural water systems in Estonia, we recorded water temperature during the 2011/2012 winter season at Piusa River Estonia, and controlled water temperature in the experimental tanks using electronic heaters.

Results

Effect of Temperature on Survival Rate

The average water temperature was 13.8°C (SD = 2.5°C). After October 10, 2011, the water temperature decreased below 10°C for a five month period. December, January and February were the coldest months, when the average water temperatures were below 5°C in both tanks (Fig. 4.1).

The first dead crayfish was found on November 21, 2011 in Tank 1 when the water temperature decreased to 0.7°C. Before January 26, 2012, there were no dead animals

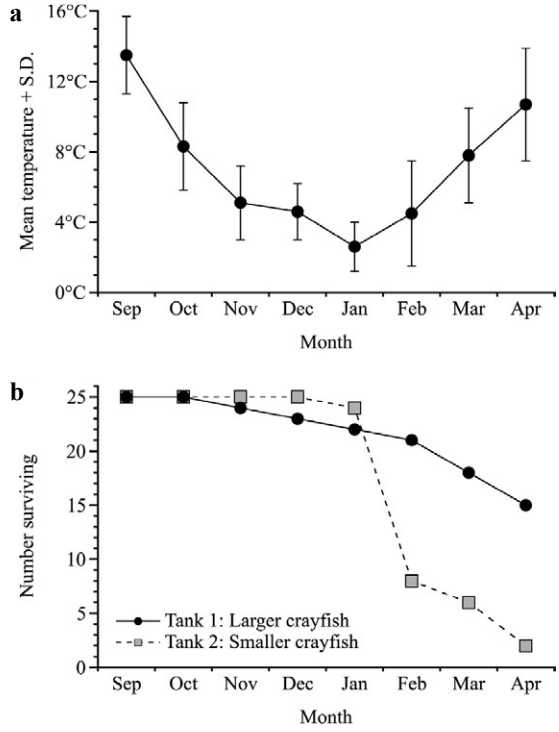

Fig. 4.1 Relationship between lower water temperature and survival of marble crayfish in Estonia, northern Europe in the 2011/2012 winter season. Marble crayfish were divided into two tanks based on body size.

in Tank 2, but the mortality increased rapidly when the water temperature was less than 2°C and the bottom of tank was iced for 16 days. Sixteen marble crayfishes died during this period. Mortality in Tank 1 increased when water temperature increased rapidly at the end of February. At the end of the experiment, the survival rate in Tank 1 was higher (60%) than in Tank 2 (8%) (Fig. 4.1).

Effect of Temperature on Growth, Reproduction and Behavior

During the experiment, two crayfish molted at temperatures below 10°C. The first was on November 9, 2011, when the water temperature had been below 10°C in Tank 2. Molts in Tank 1 were not observed.

At the start of the experiment, five marble crayfish stocked into Tank 1 carried eggs. No crayfish with eggs were stocked into Tank 2. The first dead crayfish with eggs was found on January 3, 2012. All the eggs were colored orange and covered with mold. The four remaining egg bearing could later not be identified, probably because the eggs were lost. Hatchlings were not observed. At the end of the experiment, no crayfish had eggs. When the surviving crayfish were transferred back into the indoor aquarium at a temperature of approximately 20°C, one spawned eggs within the first week, and the others 15 spawned soon after. These eggs hatched normally and their progeny survived.

Marble crayfish were active and ate at temperature above 10°C. Crayfish become less active when the water temperature decreased, and stopped eating below 10°C. Most crayfish hid in shelters at temperatures below 5°C. At temperatures below 2°C, some marble crayfish came out of the shelters and lay motionless on the bottom of tanks like dead animals, but movement could be seen when they were observed closely. When the water temperature was increased above 4°C, crayfish became more active and shelters were used less often. One crayfish had a missing claw and other one had a claw in regeneration, suggesting aggressive behavior.

Discussion

Effect of Temperature on Survival Rate

The influence of temperature on crustaceans depends on the thermal tolerance of the species, acclimatization response and physiological and behavioral adaptations (Espina et al. 1993, Gutiérrez-Yurrita 2000, Hill et al. 2004). Animals that are conformers to the environment, such as many invertebrates, face physiological and behavioral obstacles to survive in extreme environments (Chung et al. 2012). The marble crayfish is a special form closely related to *Procambarus fallax* and its natural habitat is still unknown, although *P. fallax* is a subtropical American species (Martin et al. 2010). Due to human activities, marble crayfish have been spread to several countries in the world and have successfully established populations in the wild, making it a new threat to the ecosystems in which it is introduced (Jones et al. 2009, Kawai and Takahata 2010, Kawai et al. 2009, Feria and Faulkes 2011). Karplus et al. (1998) suggest that temperate and sub-tropical crayfishes are not resistant to low temperatures, but finding marble crayfish in natural temperate waters indicates that they tolerate a wide range of environmental conditions. There is little information about marble crayfish's resistance to low water temperatures, but it can survive in ponds that freeze over during winter in France, Central Europe (Souty-Grosset et al. 2006). In Estonia, Nordic Europe, marble crayfish are a popular aquarium species. So far, fortunately, marble crayfish have not been found in the wild in Estonia.

The present experiment indicates that marble crayfish could survive for a short period (less than one week) at 1–2°C, but longer periods (more than two weeks) at low temperature (1–2°C) caused high mortality (Fig. 4.1). The differences in mortality could be caused by different sizes of crayfish in tanks, as larger crayfish seemed to be more tolerant of very low temperatures (Fig. 4.1). However, both tanks went through three months at an average water temperature below 5°C, and marble crayfish survived those harsh conditions. Water temperature varies temporally on a daily and annual cycle in nature, but water temperatures do not change rapidly under natural conditions because of high heat capacity of water.

The body temperature of aquatic invertebrates closely follows that of the environment. This means that when temperatures fluctuate, an organism has to be able to maintain its physiological functions in spite of changes in body temperature. The main mechanism to compensate for these changes in temperature is metabolic adaptation (Thoeye et al. 1987). The effect of temperature on metabolism was not measured in this study, but metabolic rate is mostly determined by water temperature (García-Guerrero

et al. 2003) and has a direct effect on survival (Thomas et al. 2001, García-Guerrero et al. 2003, Carmona et al. 2004) and growth (Meade et al. 2002, Hammond et al. 2005).

Gradual seasonal changes in temperature allow organisms to acclimate to the harsher mid-seasonal winter or summer, but an abrupt temperature change can be lethal (Somero et al. 1996). With respect to temperature tolerance range, the high temperature limit is more variable than the low temperature limit (Freitas et al. 2010). Marble crayfish are best cultured at temperatures of 18–25°C, but could withstand temperatures below 8°C and above 30°C for many weeks, although mortality increases under such conditions and reproduction stops (Vogt et al. 2004). Seitz et al. (2005) showed that most marble crayfish exposed to low temperature (8°C, 10°C) survived these conditions: only two of eight individuals died at 8°C, and the remaining six individuals survived more than 40 days (i.e., the experimental period at 8°C) and more than 100 days when they experienced temperatures of 10°C to 8°C, respectively. In our experiments, tanks experienced temperatures less than 2°C over 27 days, in addition to frequent temperature fluctuations in the 10°C range, but marble crayfish survived in both tanks. Winter water temperature in natural bodies of water in Estonia is 0–2°C, as confirmed by measurements from Piusa River, although the water temperature could be higher at greater depths, such as in lakes. We could not keep the water temperatures as consistently low as in Piusa River, so we cannot state that marble crayfish could survive in Estonian climate conditions, but our study showed that marble crayfish are very resistant to extreme temperature conditions.

Effect of Temperature on Growth

The growth pattern in crustaceans is a discontinuous process with successive molts. Temperature is the most important environmental factor influencing crayfish growth because it directly affects metabolic rate (Schmidt-Nielsen 1997, Diaz et al. 2004, Diaz-Herrera et al. 2006). Higher temperatures shorten the molt cycle and lead to faster growth (Hartnoll 2001), but only within a suitable range (García-Guerrero et al. 2003). Optimum temperatures for the growth of *Procambarus clarkii* range from 20–25°C, but growth is not drastically reduced until the temperature falls below 13°C or rises above 32°C (Aiken and Waddy 1987). Marble crayfish increase in length and weight are strongly temperature dependent, being highest at 30°C, and lowest at 15°C. For example, at 25°C, cephalothorax length and total weight increased by 17.5 mm and 1700 mg, respectively over 150 days, whereas at 15°C these parameters increased by 7 mm and 100 mg, respectively during the same period of time (Seitz et al. 2005). Seitz et al. (2005) noted that three marble crayfish molted at 10°C. Our study indicated that marble crayfish could molt at temperatures below 10°C. At least two crayfish molts were recorded while the water temperature fluctuated between 5.1°C to 9.5°C, over 30 days in Tank 2.

Effect of Temperature on Reproduction

According to Wear (1974), Heasman and Fielder (1983), Choy (1991), Zeng and Wang (1991), and Arshad et al. (2006), temperature is one of the most important factors regulating egg development for several crustacean species. The effect of temperature on gonadal development and spawning of freshwater crayfish at different temperatures are

different (Osalde et al. 2004). For example, in *P. clarkii*, embryonic development can last only 2–3 weeks (Vogt 2013), whereas in astacid crayfish, embryonic development can last 6–9 months in natural conditions (Reynolds et al. 1992).

Eleven marble crayfishes of different age and size (TL between 46–56 mm) were used in our experiment. These crayfish lived in tanks at approximately 21°C before their use in our experiments. During the experiments, water temperature decreased below 10°C, all eggs of individuals were orange, and the animals molted or died by the end of the experiment. Because marble crayfish is a warm water species, the reproductive cycle stops at temperatures below 10°C, which is confirmed by our study. There are few studies of limiting temperature of marble crayfish reproduction, but *P. clarkii* can copulate and spawn in temperatures of 10°C or lower (Cooper et al. 2011).

The native distribution of *P. fallax* in the Florida peninsula and southern Georgia (approximate 30.45°N latitude) (Hobbs 1981), where the average minimum winter air temperatures fall below 6°C (Myers and Ewel 1990) in the northern part of the range. In Estonia, marble crayfish could not reproduce all year round due to low water temperatures between autumn and spring, but during the summer, when average water temperatures are above 20°C, they could reproduce at least once per year. Rennit (2011) showed that marble crayfish started spawning when the water temperature reached 15.8°C. Both of our winter experiments showed that marble crayfish that survived low temperatures spawned soon after the experiment when the water temperature in their aquariums was increased to 20°C. Eggs hatched normally and the progeny survived.

Effect of Temperature on Behavior

Most crustaceans avoid harsh conditions by moving to an area that is more suitable for the physiological functions that ensure survival, growth and reproduction (Gonzalez et al. 2010). Crayfish can avoid some temperatures when they have a choice (Hobbs 1981, Payette and McGaw 2003). *Astacus astacus* avoid warm temperatures (> 20°C) in a 10–25°C gradient and water colder than 10°C in a cooler gradient (4–18°C) (Kivivuori 1994). This implies that crayfish, and perhaps crustaceans generally, can detect temperature, either directly through thermal sensory reception or from changes in physiological conditions due to temperature changes (Chung et al. 2012).

Our study showed that decreasing temperature changes marble crayfish behavior. Crayfish hid in shelters most of the time in both experiments. When the water temperature decreased to less than 2°C, crayfish came out of shelters and lay motionless on the bottom of tanks. Crustaceans must rely on behavioral thermoregulation, on thermal acclimation capacity, and on adopting dormant or resting stages to survive temperature extremes (Lagerspetz and Vainio 2006). In our study, marble crayfish became more active at temperatures above 6°C, but were not fed during water temperatures did not rise above 10°C and started eating at temperatures above 12°C.

Conclusions

Experiments with outdoor tanks in Estonia indicated that marble crayfish survive temperatures below 6°C for six months and tolerate temperatures below 2°C at least one week. Marble crayfish can molt at temperatures below 10°C and reproduce after surviving extreme environmental conditions. This suggests that marble crayfish might

be able to survive North European winters and to reproduce again in the following summer. Therefore it is recommended to prohibit the keeping and dealing with marble crayfish as non-native crayfish species in European countries to protect indigenous European crayfish species.

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The effect of two different feeds on growth, carapace colour, maturation and mortality in marbled crayfish (*Procambarus fallax* f. *virginalis*)

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Abstract The effect of two different feeds (30 % protein common carp feed without astaxanthin and astaxanthin-rich discus feed with 20 % shrimps and 46 % protein) on growth, carapace colour, maturation and mortality in marbled crayfish (*Procambarus fallax* f. *virginalis*) was examined under laboratory conditions. Feeding trials were carried out during 123 days at room temperature in triplicate per treatment (45 crayfish per treatment, 15 crayfish per aquarium). At the end of the trial, crayfish fed with discus feed exhibited uniformly the same dark blue carapace colouration, while crayfish fed with carp feed exhibited significantly lighter and more variable colouration in shades of grey. Growth was significantly faster ($P < 0.001$) among crayfish fed with higher protein content discus feed than among crayfish fed with lower protein content carp feed. Neither the effect of aquaria on growth nor the effect of feed on maturation and mortality were found to be significant ($P > 0.05$). Thus, our study showed that the type of feed had a significant impact on the growth and carapace colouration, but not on the maturation and mortality in the marbled crayfish.

Keywords Marbled crayfish · *Procambarus fallax f. virginalis* · Feed · Astaxanthin · Carapace colour · Growth rate

Introduction

The rapid expansion of aquaculture for the commercial production of fish and crustaceans has led to an ever-increasing demand for improved and cost-effective feeds (Boonyaratpalin et al. 2001). Carapace colour is an important quality factor for crayfish trade, both for consumption or aquarium rearing. Sommer et al. (1991) stated that the ability to market

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adult crustaceans is usually dependent on the level of their colouration. Consumers are attracted by bright and appropriate colouration, which is associated with freshness and quality of the product (Boonyaratpalin et al. 2001). Crayfish have a wide variety of colour combinations depending on species and factors such as diet, environment and genetics (Thacker et al. 1993; Ghidalia 1985; Wade 2010).

Carotenoids, particularly astaxanthin, are the primary pigments which influence crustacean shell colour (Wade et al. 2005). In the exoskeleton of live crustaceans, the orange red colour of the astaxanthin may be modified to brown, purple, green or blue through the formation of carotenoprotein complexes (Boonyaratpalin et al. 2001). The red colour of cooked crustaceans is produced by the release of the individual carotenoid prosthetic group (astaxanthin) from the carotenoproteins when denatured by the cooking heat (Ponce-Palafox et al. 2006). Animals are unable to synthesize these pigments; therefore, they must accumulate these pigments through the diet (Lee 1977; Shahidi et al. 1994; Nègre-Sadargues et al. 2000; Velu et al. 2003). The desired colouration is achieved by including in the feed astaxanthin, which is the carotenoid responsible for the natural colour (Boonyaratpalin et al. 2001).

Carotenoid pigments are largely represented in both vegetal and animal kingdoms (Petit et al. 1997). Although the biosynthesis of carotenoids can be carried out by plants, algae and bacteria, this kind of natural pigment is also found in animals such as fish and shell-fish which live in the natural environment (Rodriguez et al. 1973). Reports on specific dietary carotenoid sources in the form of astaxanthin requirement of freshwater crayfish are, however, scanty (Noverian et al. 2011).

During the last decade, the marmorkrebs or marbled crayfish (*Procambarus fallax* f. *virginalis*) has become very popular among hobby aquarists and researchers (Vogt 2008, 2010; Faulkes 2010) because of their appealing colouration, undemanding nature and exceptional mode of reproduction (Chucholl et al. 2012). The marbled crayfish reproduces parthenogenetically, and the populations consist of only females, which are genetically identical (Martin et al. 2007). These genetically identical batch mates showed surprisingly broad ranges in variation of colouration, growth, lifespan, reproduction, morphometric parameters, behaviour and fluctuating asymmetry of sense organs even when reared under identical conditions (Vogt et al. 2008). However, the effect of different types of feed on marbled crayfish shell colour, growth and reproduction has not been studied. Therefore, the aim of the present study was to assess the effect of two types of feed with different levels of dietary astaxanthin on carapace colouration, growth rate, maturation and mortality in marbled crayfish.

Materials and methods

Experimental setup

The feeding trial was set up at the Laboratory of the Department of Aquaculture, Institute of Veterinary Medicine and Animal Sciences, Estonian University of Life Sciences and lasted 123 days (from February 8 to June 10, 2013). Approximately 19-week-old marbled crayfish were all collected from a single parent. The initial size of crayfish varied from 13 to 28 mm total length (from 0.08 to 0.46 g), and four carapace colour variants (light blue, blue, light brown, darker brown) were observed (Fig. 1; Tables 1, 2).

A total of 90 crayfish were randomly allocated into six 55 L glass aquaria in triplicate per treatment (45 crayfish per treatment, 15 crayfish per aquarium) under the same rearing conditions. Each aquarium was equipped with filter and aerator, black coloured gravel on

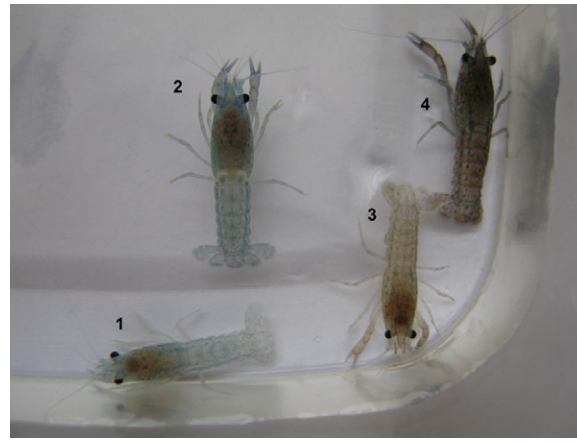

Fig. 1 Carapace colour variants of marbled crayfish at the beginning of the feeding trial—*light blue* (*1*), *blue* (*2*), *light brown* (*3*) and *darker brown* (*4*). (Color figure online)

Table 1 Number of marbled crayfish per carapace colour variant at the beginning, middle and the end of the feeding trial

Date	Feed	Light blue	Blue	Dark blue	Light brown	Darker brown	Light grey	Darker grey	Greyish brown	*P* value
08.02.13	Carp	7	9	0	8	21	0	0	0	0.0054
	Discus	9	9	0	20	7	0	0	0	
11.04.13	Carp	9	0	0	9	0	22	2	0	<0.0001
	Discus	0	37	0	0	3	0	0	0	
10.06.13	Carp	0	0	0	0	0	17	14	9	<0.0001
	Discus	0	0	35	0	0	0	0	0	

Table 2 Mean length and weight of marbled crayfish at the beginning, middle and the end of the feeding trial

Date	No. of crayfish	Feed	Mean length ± SD, mm	*P* value	Mean weight ± SD, g	*P* value
8.02.2013	45	Carp	20.4 ± 2.3	0.1098	0.18 ± 0.5	0.1313
	45	Discus	21.3 ± 2.9		0.21 ± 0.09	
11.04.2013	42	Carp	31.3 ± 1.9	<0.0001	0.70 ± 0.13	<0.0001
	40	Discus	36.3 ± 3.0		1.06 ± 0.30	
10.06.2013	40	Carp	36.3 ± 3.4	<0.0001	1.15 ± 0.31	<0.0001
	35	Discus	45.6 ± 3.3		2.45 ± 0.54	

the bottom and 15 pieces of PVC pipes that served as shelters for crayfish. Water temperature varied from 20 to 22 °C according to room temperature. Photoperiod was adjusted to natural light regime. Aquariums were cleaned once per week.

Table 3 Chemical composition of feeds used in experiment

	Alleraqua classic Common carp feed	JBL grana discus Discus feed
Composition	*Quantity*	
Protein (%)	30	46
Fat (%)	7	7
Ash (%)	5.9	7
Fibre (%)	4.5	2.5
Vitamin A (IU/kg)	10.000	25.000
Vitamin D_3 (IU/kg)	1.000	2.000
Vitamin E (mg/kg)	200	330
Vitamin C (stabil) (mg/kg)	–	400
Molluscs and crustaceans (%)	–	27.5

Feeding

The crayfish in treatment Group 1 were fed with common carp feed without astaxanthin, and the crayfish in treatment Group 2 were fed with a commercial discus feed with astaxanthin (Table 3) at a quantity 5 % of crayfish body weight once per day except weekends.

Data collection and colour determination

The crayfish were individually weighed and measured and examined for the presence of eggs at the beginning (February 8, 2013), middle (April 11, 2013) and the end (June 10, 2013) of the feeding trial. Carapace colour was examined visually in plastic box (12.8 × 9.2 × 4.0 cm) through the water with a white paper sheet as a background. Altogether eight colour variants could be discriminated: light blue, blue, dark blue, light brown, darker brown, light grey, darker grey, and greyish brown (Figs. 1, 2, 3).

Statistical analyses

The effect of feed on growth was estimated by the Student's *t* test, the effect of feed on colouration was estimated by the Fisher's exact test, and the effect of feed on mortality was estimated by odds ratio test. The effect of aquaria on growth within the treatment groups was estimated by Tukey multiple comparisons test. All data were tested for normality prior to analysis. Statistical analyses were conducted using R Statistical Software package 3.0.2.

Results

Effect of feed on colouration

At the beginning of the trial, the marbled crayfish had mostly light or darker brown and light and blue carapace colouration (Fig. 1; Table 1). After 2 months of feeding, crayfish

Fig. 2 Carapace colour variants of marbled crayfish in Group 1 (carp feed) at the end of the feeding trial—*darker grey* (*1*), *greyish brown* (*2*) and *light grey* (*3*). (Color figure online)

Fig. 3 Carapace colour variants of marbled crayfish in Group 2 (discus feed) at the end of the trial—*dark blue* (*1*). (Color figure online)

fed with astaxanthin-free carp feed (Group 1) were much lighter and more variable than crayfish fed with astaxanthin-rich discus feed (Group 2) that exhibited almost uniformly the same blue carapace colouration (Table 1). Only three crayfishes in Group 2 exhibited darker brown colouration. At the end of the trial, crayfish in Group 2 exhibited all the same dark blue carapace colouration (Fig. 3, Table 1), while the crayfish in Group 1 exhibited more variable colouration in shades of grey (Fig. 2; Table 1). The differences between the feeding groups in carapace colouration were statistically significant ($P < 0.05$) both at the middle and at the end of the feeding trial.

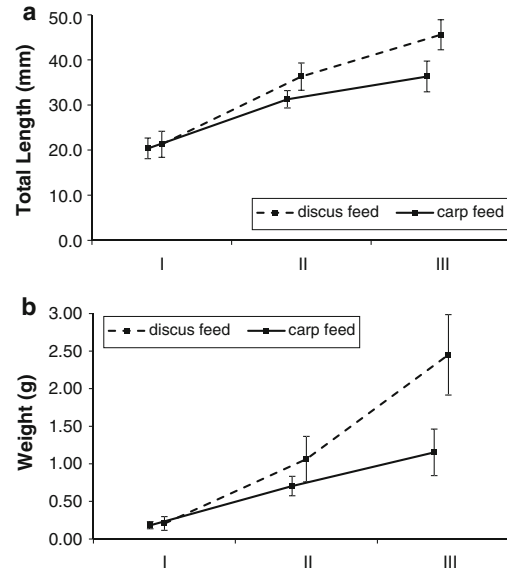

Fig. 4 Mean lengths (**a**) and weight (**b**) with SD of marbled crayfish at the beginning, middle and the end of the feeding trial

Effect of feed on growth rate

At the beginning of the experiment, there were no significant differences in the mean body weight and total length between the two experimental groups (Table 2). However, 2 months later, the crayfish in Group 2 (discus feed) were significantly larger ($P < 0.05$) than the crayfish in Group 1 (carp feed) and by the end of the trial, the differences between the two groups were even larger (Fig. 4; Table 2). During the whole feeding trial (123 days), the mean body length of Group 1 and 2 increased by 15.9–24.3 mm, respectively, and the mean body weight increased by 0.97–2.24 g, respectively. The effect of aquaria on growth was not significant within both treatment groups ($P > 0.05$).

Effect of feed on maturation

At the beginning of the trial, no crayfish with eggs were observed. In the middle of the trial, three marbled crayfish in Group 1 (carp feed) had eggs in contrast to crayfish in Group 2 (discus feed) with no eggs. At the end of the trial, six crayfish in both feeding groups (15 % in Group 1 and 17 % in Group 2) had eggs. This indicates that the type of feed had no effect on the maturation of marbled crayfish.

Effect of feed on mortality

After 2 months of feeding trial, the mortality of marbled crayfish in Group 1 and Group 2 was 7 and 11 %, respectively. At the end of the feeding trial, the mortality among crayfish fed with discus feed (Group 2) was twice as high (22 %) as the mortality among crayfish fed with carp feed (Group 1, 11 %). However, the difference was statistically not significant (odds ratio 0.5 (CI 0.156–1.604).

Discussion

Crustaceans have a wide variety of colour variations depending on species and genetic factor, moult stage, age, diet and environmental factors such as light regime and background colour (Thacker et al. 1993; Ghidalia 1985; Aiken and Waddy 1987; Parisenti et al. 2011; You et al. 2006; Beingesser and Copp 1985). In our study, all crayfish were genetically identical, had the same age and were reared under the same conditions, except the diet. It is known that astaxanthin concentration is an appropriate indicator of body colour of shrimp (Crozier 1967; Menasveta et al. 1993; Nègre-Sadargues et al. 2000; Stepnowski et al. 2004), and it is the primary pigment which influences crustacean shell colour (Wade et al. 2005). It has been shown that astaxanthin appears as a red pigment, but when complexed with various proteins, the light absorbance shifts and cause crustaceans to range in colour from green, yellow, blue to brown (Lorenz 1998). Thus, despite the fact that astaxanthin is the chromophore prosthetic group of the different carotenoproteins, many colours can be achieved (Muriana et al. 1993; Nur-E-Bordan et al. 1995; Britton et al. 1981). The results of our feeding trial showed that astaxanthin-rich feed could change the initial four carapace colour variants (light blue, blue, light brown, darker brown) to uniformly blue within 2 months from the start of feeding and the blue colour darkened uniformly over time. The crayfish which received astaxanthin-free feed had lighter and more variable colouration in shades of grey and the blue colouration disappeared all together. Our results are generally consistent with Harpaz et al. (1998) who showed that the Australian red claw crayfish (*Cherax quadricarinatus*) receiving feeds fortified with carotenoids exhibited better carapace colouration than those in the control group, which were fed a diet to which no carotenoids were added. Also, Tanaka (1978) has suggested that crustaceans should be fed diets supplemented with carotenoids for preventing the fading of their colouration. As the marbled crayfish has become very popular among hobby aquarists (Vogt 2008, 2010; Faulkes 2010), it is important to understand how different feeds affect the carapace colouration in order to develop special feeds to obtain the desired colour of crayfish.

Some studies have demonstrated a genetic basis for colour variation in several north American freshwater crayfish species (Volpe and Penn 1957; Black 1975; Black and Huner 1980), and it was found that the blue phenotype, which can be easily distinguished from the normal colour phenotype in newly released juvenile yabbies (*Cherax destructor*), was inherited as an autosomal recessive trait (Walker et al. 2000). In our experiment, we used genetically identical marbled crayfish batch mates which demonstrated variations in colour and growth even if they were reared under identical conditions. In addition, it has been shown that marbled crayfish demonstrate differences in lifespan, reproduction, morphometric traits, behaviour and fluctuating asymmetry of sense organs (Vogt et al. 2008). Vogt et al. (2007) indicated that despite being raised in the same environment, individual genotypes can map to numerous phenotypes via developmental variation, thus generating variability among clone mates and individuality in a parthenogenetic species.

Growth of crayfish has a genetic component, but is also greatly influenced by the rearing temperature and the food (Jones et al. 2000; Reynolds 2002). We can exclude the effect of genetic component and rearing temperature on growth in our experiment because all crayfish were genetically identical clone mates and were reared under identical temperature conditions. Harpaz et al. (1998) indicated that growth and survival of the crayfish were not affected by the addition of carotenoids to their diet. Our study indicated that growth was significantly faster among crayfish fed with astaxanthin-rich feed than among crayfish fed with astaxanthin-free feed. However, this can be explained by the higher protein content of

the discus feed rather than its astaxanthin content. Protein is the most expensive component in balanced feed and is probably the most important feed element for the growth of cultured species (Jones et al. 1996). An important limitation of the industry's consolidation has been the lack of appropriate information on nutrient requirements and feeding practices for the species (D'Abramo and Sheen 1996; Jones and Ruscoe 1996). Thus, there are other aspects to consider when developing the special feed for aquarium crayfish species in order to find the balance between cost and needs. Aquarium crayfish species do not need to grow fast because fast growth may cause cannibalism as indicated also by our study where the mortality was higher due to faster growth of marbled crayfish in astaxanthin-rich feeding group. However, it has been noticed that the marbled crayfish have a more peaceful nature compared to other crayfish species, having mostly a conflict-avoiding and hiding strategy (Seitz 2001; Lukhaup 2003).

The first spawning of marbled crayfish occurs on average at an age of 25 weeks when reared at 25 °C, and after 35 weeks at 20 °C (Vogt et al. 2004). In our feeding trial, we started with approximately 19-week-old marbled crayfish that were kept at a water temperature between 20 and 22 degrees. After 9 weeks of feeding, three individuals fed with astaxanthin-free feed had eggs, while the crayfish fed with astaxanthin-rich feed had no eggs. It has been reported that an unbalanced or incomplete diet causes poor reproductive performance or may even stop animals from reproducing (Izquierdo et al. 2001; Mengqing et al. 2004). During the reproduction and growth of crustaceans, the limitation of carotenoids can lead to nutritional imbalances, physiological alterations and diseases (Harrison 1990; Scott 1999). There have been few investigations of the regulating function and induction of maturation by nutritional agents such as carotenoids and retinoids, which in vertebrates are known to carry out important functions in protein expression and cellular differentiation (Liñán-Cabello et al. 2004). Studies with shrimps have indicated that supplementation with carotenoids and retinoids is important during the reproductive cycle (Liñán-Cabello et al. 2003; Liñán-Cabello and Jesús 2004). Our study showed that astaxanthin and protein-rich feed did not accelerate marbled crayfish maturation. Marbled crayfish fed without astaxanthin feed became mature earlier than crayfish fed with astaxanthin feed. However, by the end of the feeding trial, the proportion of crayfish with eggs was similar in both feeding groups. The effect of different feeds on marbled crayfish maturation warrants more specific studies in future.

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Development and characterization of novel tetranucleotide microsatellite markers in the noble crayfish (*Astacus astacus*) suitable for highly multiplexing and for detecting hybrids between the noble crayfish and narrow-clawed crayfish (*A. leptodactylus*)

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ABSTRACT

48 novel tetranucleotide microsatellite loci were developed for the noble crayfish (*Astacus astacus*) using Illumina MiSeq next generation sequencing technology. It was demonstrated that 25 loci were polymorphic and 19 loci could be co-amplified in a single multiplex polymerase chain reaction (PCR) assay and genotyped as a single panel on Applied Biosystems 3500 Genetic Analyser. The 19-plex assay was tested on 232 individuals of *A. astacus* originating from seven wild populations in Czech Republic and in Estonia. The multiplex assay designed in this study can be successfully applied in studies requiring high genetic resolution, such as population structuring, relatedness analysis, and stock identification. 21 loci were also successfully cross-amplified in the narrow-clawed crayfish (*Astacus leptodactylus*) from which 14 were polymorphic. In addition, 13 loci (both monomorphic and polymorphic) possessed species-specific allele size range in *A. astacus* and *A. leptodactylus* and can be applied for detecting possible hybrids between these sister species.

Statement of relevance: The novel 19-plex microsatellite assay can be applied for genetic management of captive stocks of the noble crayfish (selection of strains, planning of matings, avoiding of inbreeding) and in studies requiring high genetic resolution, such as parentage assessment, relatedness analysis or strain identification.

1. Introduction

The genus *Astacus* (*Astacidae*, Decapoda,) comprises three freshwater crayfish species indigenous to Europe: the noble crayfish (*Astacus astacus* L.), the narrow-clawed crayfish (*A. leptodactylus* Eschscholtz) and the thick-clawed crayfish (*A. pachypus* Rathke) (Holdich, 2002). The current distribution of *A. astacus* and *A. leptodactylus* overlaps to a great extent, though the main centre of their occurrence is in Central and Northern Europe, and in Eastern Europe and the Middle East, respectively, while *A. pachypus* is restricted to the northern coasts of the Black and Caspian Seas (Kouba et al., 2014).

The native crayfish species are more highly valued by consumers in Europe as opposed to introduced species and *A. astacus* is the most highly appreciated freshwater crayfish species in Europe (Policar and Kozák, 2015). Once abundant, it has suffered from a long-term population decline due to introduced non-indigenous species, crayfish plague, habitat loss and over-harvesting. It is now considered threatened according to the Bern convention, the EC's Habitat Directive and the IUCN Red List (Edsman et al., 2010). *A. astacus* are mainly cultured in the Nordic and Baltic countries and in the Alpine region (Skurdal and Taugbøl, 2002). They are mainly produced for stocking into natural waters, and to some extent, for consumption using extensive production methods in natural or man-made ponds or semi-intensive cultivation technology in ponds and raceways though more recently, intensive rearing methods in recirculating aquaculture systems have also been tested (Ackefors, 2000; Seemann et al., 2015). However, it is very difficult to obtain reliable information about current production volumes. Official FAO statistics report aquaculture production of *A. astacus* in Europe from three countries (Estonia, Bulgaria and Ukraine) with total production volumes ranging from only 0.7 to 13.3 t/year during the period 2005–2014 (FAO, 2016). However, it is known that *A. astacus* has also been produced in variable amounts for consumption in other countries (e.g. in Sweden and in Finland), and according to Ackefors (1998), the total aquaculture production of *A. astacus* in Europe in 1994 was about 27 t.

Until recent times, knowledge of intra-specific genetic diversity of *A. astacus* was rather limited and the genetic background of crayfish has been rarely considered when selecting material for re-introduction, supplemental stocking or captive breeding. Similarly, captive broodstocks of *A. astacus* have been managed without any knowledge about the levels of genetic variability, differentiation and inbreeding or the degree of

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Table 1
Characterization of tetranucleotide microsatellite loci in eight individuals of *A. astacus* and *A. leptodactylus*. Loci shown are only those successfully amplified in one or both species.

Locus	Repeat motif	Label of the M13 primer	Forward primer sequence 5′ → 3′[a]	Reverse primer sequence 5′ → 3′	*A. astacus*			*A. leptodactylus*		Suitability for hybrid detection	GenBank accession no.
					No. of alleles	Expected allele size (bp)[a]	Observed allele size range (bp)[a]	No. of alleles	Observed allele size range (bp)[a]		
Aast4_2	ACCT	6FAM	M13-AACCCATCCCTCCTACGTCT	GGAGGGACATTGAATAACGG	2	115	106–110	2	176–184	Yes	KU955535
Aast4_3	ACGC	6FAM	M13-TTTCCTGTCTTTCGGCTGTC	ACCCTGCAAGCACATCTAGG	2	120	115–119	4	160–167	Yes	KU955536
Aast4_5	AATG	6FAM	M13-CCCACTTTAAGCAGAAGACACA	ATGATTGCCGAGTTCCTTGT	1	141	137	2	121–125	Yes	KU955538
Aast4_7	ACAT	6FAM	M13-ATGAAGCTTGAGAAGGCCTATG	TGGTTAACATAACTGATGTGGGA	2	159	156–168	1	158	No	KU955540
Aast4_8	ACAG	6FAM	M13-CCATCACTGGAAATACGGAAA	AAGCGTCTGGCCTCCAAC	1	164	163	2	129–133	Yes	KU955541
Aast4_10	ACAT	6FAM	M13-TACAGTGGTCTTCGTGCGTG	AGTGGCAAATCAGGAGAACG	5	181	165–189	4	140–177	No	KU955543
Aast4_12	AATG	6FAM	M13-CCCACTTTAAGCAGAAGACACA	CACCACTAAATCCTTATACAAATCCA	1	189	187	2	171–175	Yes	KU955545
Aast4_14	ACAT	VIC	M13-CGGCCAGGAATGTTATGTTT	GCATTTGGAACATGTTTGCT	1	191	192	0	No amp.	No	KU955547
Aast4_16	ACGC	VIC	M13-GGTGTCTCGCGAAATGTAAA	TAGATGAATGGGCAGGAAGC	2	207	199–203	3	176–182	Yes	KU955549
Aast4_17	ACGC	VIC	M13-CCGCGCTAGTAAACTTGTCC	GTTGGTTGTGAGCCGACAGT	4	207	190–233	0	No amp.	No	KU955550
Aast4_18	ACAG	VIC	M13-TCTACATCCTTATCTGGTGTTTCA	GTGCCGCATGTTCCAGAAT	3	209	206–214	2	207–211	No	KU955551
Aast4_19	ACAT	VIC	M13-TTGTTTCGTGTTAATTCACTTTGG	CATCCTCTCGCTTCAGTTGC	3	209	205–213	0	No amp.	No	KU955552
Aast4_20	AGCC	VIC	M13-CGTTACCCTTCCATATTGGC	TTATAAATGTCTGCAGCTCCC	3	209	193–205	5	166–268	No	KU955553
Aast4_22	AAGG	VIC	M13-GGAGAAATGGATCAGCTACGA	ACATCATAGGTATGTTGCTTGGG	2	213	211–215	1	211	No	KU955555
Aast4_23	AGAT	VIC	M13-GGGCTTAATTGATTTGCACG	AATCTCTTAGTCGGAATGTCGAT	0	213	No amp.	1	122	No	KU955556
Aast4_24	AGGC	VIC	M13-CATTAAGCAAGAAGGACGGAT	CCCACTGTTTCTCCTCTCCT	4	213	210–226	1	308	Yes	KU955557
Aast4_25	ACAG	NED	M13-CCACTCCTGTGGTTAGGTCA	CCAGGTCTTTGTCTTGTCATCA	1	217	217	0	No amp.	No	KU955558
Aast4_26	AGGC	NED	M13-TTCTGGTGGATGACATGAGC	TGCGCGATAAAGTTGAAAGA	3	224	220–232	2	249–258	Yes	KU955559
Aast4_27	ACGC	NED	M13-TCGGTCTAACAAATGCTGTG	TGTCTGCATTTATGTTGCTCG	1	225	306	0	No amp.	No	KU955560
Aast4_30	ACCT	NED	M13-TCAATTTGTTGTGATGCCAGA	CTGCATGACGCTGCTACTTT	5	253	238–254	2	144–152	Yes	KU955563
Aast4_31	AGCC	NED	M13-CCCTTCTGTCTCCTGCCAGT	GACTGATTGGCTGTCTGCTG	1	254	250	0	No amp.	No	KU955564
Aast4_32	ACAT	NED	M13-ACCTATGTTTGTTGTCTATATGGATCT	CGCCCTCCCTCAATATAGC	2	256	255–259	1	234	Yes	KU955565
Aast4_33	AGGC	NED	M13-TCCCATTACAATAATAAGTAGGCAA	CTGGGAGGGAAGTGGAGTG	0	259	No amp.	4	355–379	No	KU955566
Aast4_34	ACTC	NED	M13-TTCAAATGTAGATATCAGAGAACCCA	TGATACTCAAATTAGGAAAGAGTGGA	3	259	252–260	1	240	Yes	KU955567
Aast4_35	ACAG	NED	M13-CGTTCTCAACACGCAAACTT	CAGACCTTGAGCATGACCGT	2	260	258–262	0	No amp.	No	KU955568
Aast4_37	ACAT	PET	M13-TGGCTAAGACCAGATCTCAACA	TCTGGCACTGAAGGAGTTCT	2	265	266–270	0	No amp.	No	KU955570
Aast4_38	ACAT	PET	M13-TGTTAACCGATTGGAGGGTC	TCATATTCCAATTCCGCCTC	2	266	268–276	0	No amp.	No	KU955571
Aast4_39	ACCT	PET	M13-TTTGAGAGCCTGGGTTTCC	AAATTTACCTGAGGGCCACC	3	271	271–316	0	No amp.	No	KU955572
Aast4_40	ACAT	PET	M13-TGCAAGTAATGCATGTTAGGAA	CCTCAAACTCACAGTGCTGC	1	274	274	1	241	Yes	KU955573
Aast4_41	ACGC	PET	M13-ACTCTGTAGGCTCTGGTTTACAA	TCATACGTCAGACTGCGAGC	1	277	279	0	No amp.	No	KU955574
Aast4_42	AGGC	PET	M13-AAGCCACTAATATGCAGTCATTT	GAAATTTAATATCCATCGCCC	3	279	270–282	0	No amp.	No	KU955575
Aast4_43	ACAT	PET	M13-GGGAACGAAGAAGTCTTTACCA	TTGGATTGGGAGTGAGGTCC	2	280	276–371	3	300–308	No	KU955576
Aast4_44	ACAG	PET	M13-GGAACATTCTTTATTGGCAGG	TCACCTACCCAACATGTCTCA	4	310	311–335	0	No amp.	No	KU955577
Aast4_46	ACAT	PET	M13-AGTCAAACAAGCCTAGCACG	TGAGTACAACGCTAAAGTTAATATGTC	3	310	299–307	0	No amp.	No	KU955579
Aast4_47	ACGC	PET	M13-GCGTCGTCAAGGATACACAA	TAGACGGCGATCACTATGGC	2	315	316–320	0	No amp.	No	KU955580
Aast4_48	ACAT	PET	M13-GCAATTTCAAACAGGCTTCA	CCCGCCTTTAAATGTTTAGC	2	319	311–319	3	287–295	Yes	KU955581

[a] With 19 bp M13-tail (CACGACGTTGTAAAACGAC).

relatedness among individuals. The first microsatellite markers for *A. astacus* (a total of 19 dinucleotide repeat loci) were developed by Kõiv et al. (2008, 2009) and have been applied subsequently in several studies of genetic diversity (Gross et al., 2013; Schrimpf et al., 2014; Blaha et al., 2016). However, the number of practically usable loci in these studies was rather low (from six to 10 loci), because many loci failed to amplify consistently or exhibited extensive stuttering that made their reliable genotyping difficult. For *A. leptodactylus*, there are currently no published microsatellite loci available, and the information about intra-species genetic diversity is based on mitochondrial DNA only (Maguire et al., 2014; Khoshkholgh and Nazari, 2015).

The aim of the current study was to develop a set of polymorphic tetranucleotide repeat microsatellite markers for *A. astacus* that can be reliably and efficiently genotyped in a highly multiplexed panel. The loci were also tested for cross-amplification success in *A. leptodactylus* and screened for the presence of species-specific alleles in *A. astacus* and *A. leptodactylus* that can be applied for identification of potential hybrids between these sister species.

2. Material and methods

2.1. Genomic library preparation and sequencing

Genomic DNA was isolated from the leg muscle tissue of three adult specimens of *A. astacus* (collected from Lake Pangodi, Estonia) using High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Germany). *Hin*fI restriction enzyme digested and purified genomic DNA was prepared for sequencing using ClaSeek Library Preparation Kit (Thermo Fisher Scientific Inc, USA) and Illumina TruSeq DNA LT Sample Prep kit (Illumina, San Diego, USA). The libraries were quantified with Qubit 2.0 Fluorometer (Invitrogen, Grand Island, USA) and TapeStation 2200 (Agilent Technologies, Santa Clara, USA), validated by qPCR using Kapa Library Quantification Kit (Kapa Biosystems, Woburn, USA) and sequenced on Illumina MiSeq flowcell with 2 × 300 bp paired-end reads using MiSeq Reagent Kit v3 (Illumina, San Diego, USA). Paired-end reads were stitched with PEAR using default settings (Zhang et al., 2014).

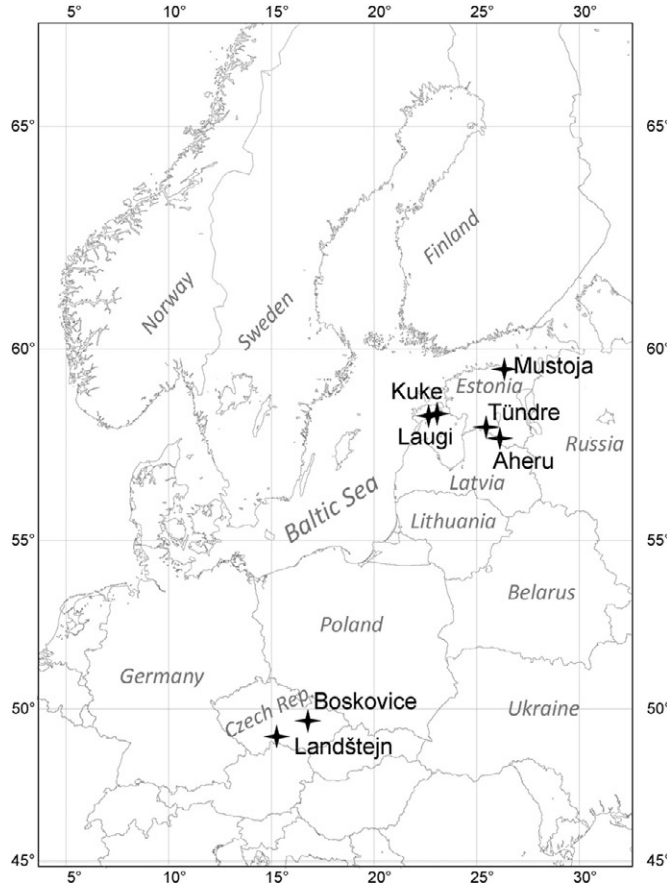

Fig. 1. Sampling locations of the *A. astacus* populations.

2.2. Detection of microsatellite motifs and initial testing of loci for amplification success and polymorphism in A. astacus and A. leptodactylus

For microsatellite motif detection, sequence selection and PCR primer design a software QDD version 3.1 was used with default settings (Meglécz et al., 2014). All sequences from the three specimens that contained tetranucleotide repeats were pooled and subjected to PCR primer design. A total of 48 primer pairs were selected according to the criteria of Meglécz et al. (2014) and were initially tested for amplification success and polymorphism in eight individuals of *A. astacus* (four individuals from water reservoirs — Boskovice and Landštejn in Czech Republic, the Danube-Black Sea catchment and one individual from each of the four different wild populations in Estonia, the Baltic Sea catchment) and in eight individuals of *A. leptodactylus* (four individuals each from Korana and Mrežnica populations in Croatia). Forward primers were designed with a 19 bp M13 tail that was labeled during the PCR reaction using a universal fluorecently (either 6-FAM, NED, PET or VIC) labeled M13 primer (CACGACGTTGTAAAACGAC). The PCR reaction (10 μl) contained 1× Type-it Multiplex PCR Master Mix (QIAGEN, Germany), 100 nM of forward primer, 200 nM of M13 and reverse primer, and ~50 ng of DNA template. Touchdown program was used for PCR amplification: initial activation of 5 min at 95 °C, followed by 20 cycles of 30 s at 95 °C, 90 s at 60 °C, 30 s at 72 °C, with the annealing temperature decreasing 0.5 °C per cycle, followed by 10 cycles of 30 s at 95 °C, 90 s at 50 °C, 30 s at 72 °C and a final extension for 30 min at 60 °C. Electrophoresis was performed using Applied Biosystems 3500 Genetic Analyser (Life Technologies, USA) and the length of fragments was determined using internal GeneScan 600 LIZ Size Standard v2.0 (Life Technologies, USA) and GeneMapper 5 software (Life Technologies, USA).

2.3. Design of the highly multiplex microsatellite panel and test for variability of loci in A. astacus

From the polymorphic loci of the intial test, 19 were further selected for multiplexing in a single PCR reaction based on the range of detected alleles (Table 1) and this 19-plex panel was tested for variability of loci in 232 individuals of *A. astacus* that originated from two wild populations from the Czech Republic (water reservoirs Boskovice and Landštejn, the Danube-Black Sea catchment) and five wild populations from Estonia (streams Kuke, Laugi and Mustoja, lakes Aheru and Tündre, the Baltic Sea catchment) (Fig. 1). Forward primers were labeled fluorecently (either 6-FAM, ATTO 550, ATTO 565 or Yakima Yellow, see Table 2). The PCR reaction (10 μl) contained 1× Type-it Multiplex PCR Master Mix (QIAGEN, Germany), 200 to 400 nM of each primer (Table 2), and ca 50 ng of DNA template. Touchdown program was used for PCR amplification as described above. This 19-plex marker panel was genotyped in a single run of an Applied Biosystems 3500 Genetic Analyser (Life Technologies, USA) and microsatellite genotypes were scored using GeneMapper v.5 software (Life Technologies, USA). GENEPOP v. 3.3 (Raymond and Rousset, 1995a) was used to test genotypic distributions for conformance to Hardy-Weinberg (HW) expectations and to test the loci for genotypic disequilibria. All probability tests were based on the Markov chain method (Guo and Thompson, 1992; Raymond and Rousset, 1995b) using 1000 de-memorization steps 100 batches and 1000 iterations per batch. Sequential Bonferroni adjustments (Rice, 1989) were applied to correct for the effect of multiple tests. MICRO-CHECKER 2.2.3 (Van Oosterhout et al., 2004) was used to assess the potential presence of genotyping errors due to scoring of stutter peaks, large allele dropouts and null alleles.

3. Results and discussion

3.1. Development of the multiplex assay and variability of tetranucleotide microsatellite loci in A. astacus

A total of 13,490,148 paired-end reads were obtained using Illumina MiSeq System and 5686 reads (0.042%) contained tetranucleotide repeats. Of the total 48 loci that were selected for initial testing, 35 amplified successfully and 25 were polymorphic among eight individuals of *A. astacus* (Table 1). From the 25 polymorphic loci, 19 were further selected for multiplexing based on their observed allele ranges and were successfully co-amplified in a single 19-plex PCR reaction. Among 232 individuals of *A. astacus* from two wild populations from the Czech Republic and five wild populations from Estonia, a total of 86 alleles were detected. The number of alleles per locus ranged from two (Aast4_38) to eight (Aast4_20) and the observed heterozygosity varied from 0.026 (Aast4_32) to 0.438 (Aast4_46) (Table 2). All 19 loci conformed to linkage and Hardy-Weinberg equilibria in all studied populations (Table 3) and in the majority of cases (131 locus-sample combinations out of 133 tests) none of the loci showed evidence for a null allele, except loci Aast4_10 and Aast4_37 in Landštejn population (data not shown).

The variability of loci as measured by the number of alleles (A) and the observed (H_o and expected (H_e) heterozygosities varied considerably among the studied populations (Table 3). The mean level of observed

Table 2
PCR conditions and variability of tetranucleotide microsatellite loci of the 19-plex microsatellite assay in 232 individuals from seven populations of *A. astacus*.

Locus name	Fluorescent label of the F-primer	Alternative fluorescent label of the F-primer[a]	Conc. F-primer (nM)	Conc. R-primer (nM)	No. of observed alleles	Observed allele size range (bp)	Observed hetero-zygosity
Aast4_2	6FAM	6FAM	200	200	4	87–99	0.168
Aast4_7	6FAM	6FAM	200	200	3	136–148	0.052
Aast4_17	6FAM	6FAM	200	200	7	170–214	0.280
Aast4_42	6FAM	6FAM	200	200	7	236–260	0.233
Aast4_46	6FAM	6FAM	200	200	4	278–290	0.438
Aast4_20	ATTO 550	NED	200	200	8	165–197	0.241
Aast4_32	ATTO 550	NED	200	200	3	236–244	0.026
Aast4_38	ATTO 550	NED	200	200	2	248–252	0.085
Aast4_48	ATTO 550	NED	400	400	3	292–304	0.142
Aast4_16	ATTO 565	PET	200	200	4	180–192	0.091
Aast4_26	ATTO 565	PET	200	200	7	195–223	0.332
Aast4_35	ATTO 565	PET	200	200	2	228–244	0.099
Aast4_47	ATTO 565	PET	200	200	4	275–299	0.220
Aast4_3	Yakima Yellow	VIC	400	400	3	91–99	0.203
Aast4_10	Yakima Yellow	VIC	200	200	7	146–170	0.267
Aast4_19	Yakima Yellow	VIC	200	200	5	181–201	0.095
Aast4_30	Yakima Yellow	VIC	300	300	6	219–239	0.412
Aast4_37	Yakima Yellow	VIC	200	200	3	239–247	0.126
Aast4_44	Yakima Yellow	VIC	300	300	4	289–313	0.083

[a] Applied Biosystems.

Table 3
Summary of variation of 19 tetranucleotide microsatellite loci in the multiplex assay of seven *A. astacus* populations: number of alleles per locus (A), observed heterozygosity (H_o), expected heterozygosity (H_e), significance of deviations from Hardy–Weinberg equilibrium (P_{HWE}).

Locus	Parameter	Boskovice	Landštejn	Aheru	Kuke	Laugi	Mustoja	Tündre
		$n = 28$	$n = 27$	$n = 34$	$n = 44$	$n = 23$	$n = 27$	$n = 49$
Aast4_2	A	2	3	2	2	2	1	2
	H_o	0.214	0.370	0.029	0.364	0.217	0.000	0.020
	H_e	0.195	0.360	0.029	0.439	0.198	0.000	0.020
	P_{HWE}	NS	NS	NS	NS	NS	–	–
Aast4_7	A	3	3	1	1	1	1	1
	H_o	0.286	0.148	0.000	0.000	0.000	0.000	0.000
	H_e	0.259	0.207	0.000	0.000	0.000	0.000	0.000
	P_{HWE}	NS	NS	–	–	–	–	–
Aast4_17	A	2	3	3	3	2	2	6
	H_o	0.214	0.519	0.118	0.182	0.261	0.407	0.327
	H_e	0.249	0.469	0.167	0.169	0.232	0.465	0.359
	P_{HWE}	NS	NS	NS	NS	NS	NS	NS
Aast4_42	A	5	4	2	1	1	3	4
	H_o	0.286	0.259	0.412	0.000	0.000	0.292	0.347
	H_e	0.349	0.294	0.332	0.000	0.000	0.382	0.363
	P_{HWE}	NS	NS	NS	–	–	NS	NS
Aast4_46	A	3	3	1	3	3	2	3
	H_o	0.464	0.444	0.000	0.523	0.684	0.474	0.521
	H_e	0.551	0.511	0.000	0.584	0.562	0.371	0.468
	P_{HWE}	NS	NS	–	NS	NS	NS	NS
Aast4_20	A	4	4	6	2	1	4	4
	H_o	0.464	0.296	0.294	0.023	0.000	0.259	0.347
	H_e	0.440	0.299	0.271	0.023	0.000	0.344	0.310
	P_{HWE}	NS	NS	NS	NS	–	NS	NS
Aast4_32	A	1	1	2	1	1	2	2
	H_o	0.000	0.000	0.059	0.000	0.000	0.074	0.041
	H_e	0.000	0.000	0.058	0.000	0.000	0.073	0.040
	P_{HWE}	–	–	NS	–	–	NS	NS
Aast4_38	A	2	2	2	2	2	1	2
	H_o	0.036	0.038	0.029	0.114	0.050	0.000	0.204
	H_e	0.036	0.111	0.086	0.108	0.050	0.000	0.217
	P_{HWE}	NS	NS	NS	NS	NS	–	NS
Aast4_48	A	2	2	1	2	2	1	2
	H_o	0.067	0.200	0.000	0.436	0.167	0.000	0.022
	H_e	0.067	0.189	0.000	0.373	0.167	0.000	0.022
	P_{HWE}	NS	NS	–	NS	NS	–	NS
Aast4_16	A	4	4	1	1	2	1	1
	H_o	0.321	0.407	0.000	0.000	0.043	0.000	0.000
	H_e	0.338	0.352	0.000	0.000	0.043	0.000	0.000
	P_{HWE}	NS	NS	–	–	NS	–	–
Aast4_26	A	5	4	3	2	2	2	3
	H_o	0.500	0.481	0.118	0.432	0.304	0.037	0.388
	H_e	0.516	0.421	0.114	0.447	0.372	0.037	0.352
	P_{HWE}	NS	NS	NS	NS	NS	NS	NS
Aast4_35	A	2	2	1	1	1	1	3
	H_o	0.393	0.259	0.000	0.000	0.000	0.000	0.102
	H_e	0.399	0.230	0.000	0.000	0.000	0.000	0.099
	P_{HWE}	NS	NS	–	–	–	–	NS
Aast4_47	A	1	1	3	3	1	1	2
	H_o	0.000	0.000	0.286	0.561	0.000	0.000	0.087
	H_e	0.000	0.000	0.309	0.559	0.000	0.000	0.084
	P_{HWE}	–	–	NS	NS	–	–	NS
Aast4_3	A	2	2	2	2	2	1	3
	H_o	0.500	0.111	0.088	0.409	0.217	0.000	0.082
	H_e	0.444	0.171	0.086	0.329	0.198	0.000	0.080
	P_{HWE}	NS	NS	NS	NS	NS	–	NS
Aast4_10	A	3	5	2	3	2	1	4
	H_o	0.429	0.407	0.147	0.455	0.087	0.000	0.245
	H_e	0.480	0.599	0.138	0.413	0.085	0.000	0.222
	P_{HWE}	NS	NS	NS	NS	NS	–	NS
Aast4_19	A	1	4	3	1	1	1	3
	H_o	0.000	0.556	0.118	0.000	0.000	0.000	0.061
	H_e	0.000	0.670	0.114	0.000	0.000	0.000	0.099
	P_{HWE}	–	NS	NS	–	–	–	NS
Aast4_30	A	4	4	4	3	2	2	5
	H_o	0.464	0.654	0.353	0.523	0.278	0.136	0.367
	H_e	0.416	0.563	0.314	0.469	0.246	0.130	0.425
	P_{HWE}	NS	NS	NS	NS	NS	NS	NS
Aast4_37	A	1	3	2	3	2	1	3
	H_o	0.000	0.231	0.061	0.364	0.050	0.000	0.061
	H_e	0.000	0.411	0.060	0.334	0.050	0.000	0.060
	P_{HWE}	–	NS	NS	NS	NS	–	NS

Table 3 (*continued*)

Locus	Parameter	Boskovice	Landštejn	Aheru	Kuke	Laugi	Mustoja	Tündre
		$n = 28$	$n = 27$	$n = 34$	$n = 44$	$n = 23$	$n = 27$	$n = 49$
Aast4_44	A	2	3	1	1	1	1	2
	H_o	0.095	0.125	0.000	0.000	0.000	0.000	0.250
	H_e	0.177	0.121	0.000	0.000	0.000	0.000	0.221
	P_{HWE}	NS	NS	–	–	–	–	NS

NS — deviation from Hardy–Weinberg equilibrium is not significant ($P > 0.05$).

genetic diversity over 19 microsatellite loci also varied considerably between populations: it was the lowest in Mustoja (proportion of polymorphic loci $P = 0.368$, $A = 1.5$, $H_o = 0.088$) and Laugi ($P = 0.579$, $A = 1.6$, $H_o = 0.124$) populations from Estonia and the highest in Landštejn ($P = 0.895$, $A = 3.0$, $H_o = 0.290$) and Boskovice ($P = 0.789$, $A = 2.6$, $H_o = 0.249$) populations from the Czech Republic (Table 4). All seven *A. astacus* populations in this study were also part of the study by Gross et al. (2013) using 10 dinucleotide repeat microsatellite markers and the overall ranking of these populations based on the mean level of genetic variability was very similar (Table 4). However, the variability of dinucleotide microsatellite loci in the same populations was generally higher (Table 4). This can be explained by higher mutation rates of dinucleotide loci in comparison with tetranucleotide loci as also shown in earlier studies of other organisms (Chakraborty et al., 1997; Kruglyak et al., 1998; Schug et al., 1998; Lee et al., 1999: Kelkar et al., 2008). Also, dinucleotide repeats often show one or more "stutter" peaks arising from multiple PCR products derived from the same reaction template that are typically shorter by one or a few repeats than the full-length product (Chambers and MacAvoy, 2000). This can result in mis-scoring of alleles and genotypes that may lead to biased estimates of the true number of alleles and the heterozygosity, especially when the lengths of two alleles in a heterozygous individual differ by only a single repeat (Ginot et al., 1996; Ewen et al., 2000; Hoffmann and Amos, 2005). In contrast, tetranucleotide repeats appear to be significantly less prone to exhibiting stutter peaks (Edwards et al., 1991; Nater et al., 2009) and as a result, their alleles and genotypes can be scored much more reliably which is crucially important for applications like forensics and paternity assignment.

3.2. Cross-species amplification in *A. leptodactylus* and identification of potential hybrids between *A. astacus* and *A. leptodactylus*

The 48 loci were also tested on eight individuals of *A. leptodactylus* from Croatia. 21 loci cross-amplified successfully, from which 14 were polymorphic possessing from two to five alleles (Table 1). The usefulness of these polymorphic loci for genetic studies of *A. leptodactylus* has to be tested on larger sample sizes.

Table 4
Average level of variation of tetranucleotide versus dinucleotide microsatellite loci in studied populations of *A. astacus*: number of individuals (n), proportion of polymorphic loci (P), average number of alleles per locus (A), average observed heterozygosity (H_o), average expected heterozygosity (H_e).

Parameter of variation	Boskovice	Landštejn	Aheru	Kuke	Laugi	Mustoja	Tündre
19 tetranucleotide repeat loci (*this study*):							
n	28	27	34	44	23	27	49
P	0.789	0.895	0.684	0.632	0.579	0.368	0.895
A	2.6	3.0	2.2	1.9	1.6	1.5	2.9
H_o	0.249	0.290	0.111	0.231	0.124	0.088	0.183
H_e	0.259	0.315	0.109	0.224	0.116	0.095	0.181
10 dinucleotide repeat loci Gross et al. (2013):							
n	30	30	24	32	29	30	24
P	1.000	1.000	0.900	0.600	0.600	0.800	0.900
A	4.3	4.3	2.7	2.2	2.2	2.1	3.8
H_o	0.506	0.439	0.229	0.208	0.195	0.193	0.303
H_e	0.513	0.450	0.237	0.216	0.167	0.207	0.311

In addition, 13 loci (both monomorphic and polymorphic) possessed species-specific allele size range in *A. astacus* and *A. leptodactylus* (Table 1) and can be potentially applied for detecting and confirming possible hybrids between these sister species. Hybrids between *A. astacus* and *A. leptodactylus* have been obtained under laboratory conditions (Furrer et al., 1999) and their occurrence in the wild has been suggested based on morphological studies (Maguire et al., 2013). However, identification of hybrids based on morphological traits is less reliable than by using bi-parentally inherited diagnostic genetic markers like microsatellites. Again, practical usefulness of these 13 microsatellite loci for detecting and confirming potential hybrids has to be tested on larger sample sizes of both species. It is quite probable that the real allele size range for each species will be significantly larger, especially after sampling from broader geographical area, and may partially overlap in *A. astacus* and *A. leptodactylus*.

3.3. Perspectives and aquaculture applications

The principles, potential power, requirements, advantages, and disadvantages of various types of genetic markers, along with their applications in a variety of aquaculture studies have been extensively discussed by Ferguson and Danzmann (1998); Davis and Hetzel (2000); Fjalestad et al. (2003); Liu and Cordes (2004) and Chistiakov et al. (2006). The major advantages and strengths of microsatellite markers are their codominant inheritance, abundance and even distribution in genome, small locus size, high polymorphism, and relatively low genotyping costs. The main challenge in the past was the high cost and large effort of developing microsatellite markers for each species using traditional molecular genetic methods (Ostrander et al., 1992). However, this has been alleviated now with the advent of next-generation sequencing, which allows the detection and characterization of large numbers of microsatellite loci in any species much more efficiently (Guichoux et al., 2011). Results of the current study confirm this excellently. Using the Illumina MiSeq System we developed 25 new polymorphic tetranucleotide repeat microsatellite markers for *A. astacus* and demonstrated that 19 markers can be cost-efficiently co-amplified in a single multiplex PCR reaction and subsequently genotyped using a single run of an Applied Biosystems 3500 Genetic Analyser. However, validation of this 19-plex microsatellite panel for genetic studies of *A. astacus* in the whole area of their distribution should be done by sampling crayfish from a broader geographical area as it is possible that the allele size ranges may be larger in populations from other regions than those observed in this study. As a result, the allele size ranges at some loci with the same fluorescent label may partially overlap and the 19-plex panel must be modified accordingly or divided into two sub-panels for multiplexing.

It has been demonstrated convincingly that the application of microsatellites (and also other DNA markers) in aquaculture has allowed significant progress in investigations of genetic variability and inbreeding in broodstocks, parentage assignments and pedigree structure, species, strain and hybrid identification, mapping economically important quantitative traits and marker-assisted selection (Davis and Hetzel, 2000; Fjalestad et al., 2003; Liu and Cordes, 2004; Chistiakov et al., 2006). There are captive *A. astacus* broodstocks in several countries in Europe (e.g. Sweden, Finland, Estonia, Latvia, Lithuania, Norway, France, Germany, Czech Republic) that have been established

for aquaculture and/or stocking material production (Ackefors, 1997; Mackevieienee et al., 1997; Arens and Taugbøl, 2005; Paaver and Hurt, 2009; Policar and Kozák, 2015). Broodstocks are usually established and supplemented by crayfish originating from local natural waterbodies. Improper management of the broodstocks may result in reduced genetic diversity and increased inbreeding. Genetic characterization and monitoring of broodstocks using microsatellite markers is therefore essential for evaluating the levels of genetic variability, differentiation and inbreeding and for determining the degree of relatedness among individuals. This allows more efficient management of broodstocks and reduces the risk of adverse changes in the gene pools of reared *A. astacus* stocks. Also, all other common applications of microsatellites (see above) could significantly facilitate the increase of aquaculture production of this highly valued crayfish species in Europe.

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Tähti Tobreluts,	Master's Degree, 2016, Katrin Kaldre (supervisor). Fishing tourism in Estonia. EULS.
Kirsi Mekk,	Master's Degree, 2014, (supervisor) Katrin Kaldre. PIT tagging effect on noble crayfish (*Astacus astacus* L.) survival. EULS
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Anton Meženin,	Master's Degree, 2013, (supervisor) Katrin Kaldre. Survival of marble crayfish (*Procambarus fallax* f. *virginalis*) at low temperature (1–40 °C)". EULS

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Juhendatud väitekirjad

Raimo Pajusalu,	magistrikraad, 2017, (juh) Ene Saadre; Peeter Padrik; Katrin Kaldre. Atlandi lõhe (*Salmo salar* L.) niisa sügavkülmutamise tehnoloogia rakendamine RMK Põlula kalakasvatuskeskuses. Eesti Maaülikool
Tähti Tobreluts,	magistrikraad, 2016, (juh) Katrin Kaldre. Kalaturism Eestis. Eesti Maaülikool
Kirsi Mekk,	magistrikraad, 2014, (juh) Katrin Kaldre. PIT märgistamise mõju jõevähi (*Astacus astacus* L.) ellujäävusele. Eesti Maaülikool
Kerli Haugjärv,	magistrikraad, 2014, (juh) Katrin Kaldre. Kahe erineva sööda mõju marmorvähi (*Procambarus fallax* f. *virginalis*) kasvule, värvusele, suguküpsuse saabumisele ning surevusele. Eesti Maaülikool
Anton Meženin,	magistrikraad, 2013, (juh) Katrin Kaldre. Marmorvähi (*Procambarus fallax* f. *virginalis*) ellujäämus madalatel temperatuuridel (1–4 °C). Eesti Maaülikool

LIST OF PUBLICATIONS

1.1. Articles indexed by Thomson Reuters Web of Science /
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Kaldre, K., Paaver, T., Hurt, M., Grandjean, F. (2017). First records of the non-indigenous signal crayfish (*Pacifastacus leniusculus*) and its threat to noble crayfish (*Astacus astacus*) populations in Estonia. *Biological Invasions*. s10530-017-1496-z.

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Artiklid/peatükid lisas loetletud kirjastuste välja antud kogumikes (kaasa arvatud Thomson Reuters Book Citation Index, Thomson Reuters Conference Proceedings Citation Index, Scopus refereeritud kogumikud)

Kaldre, K., Meženin, A., Paaver, T., Kawai, T. (2015). A Preliminary Study on the Tolerance of Marble Crayfish *Procambarus fallax* f. *virginalis* to Low Temperature in Nordic Climate. In: Tadashi Kawai, Zen Faulkes, Gerhard Scholtz (Ed.). Freshwater Crayfish: A Global Overview (54–62). Taylor & Francis.

5.2. Conference abstracts that do not belong in section 5.1. /
konverentsiteesid, mis ei kuulu valdkonda 5.1

Kaldre, K., Paaver, T., Hurt, M. (2017). Jõevähi varude seisundit ja tulevikku ohustavad faktorid Eestis - vähikatk ja invasiivsed võõrliigid. *Terve Loom ja Tervislik Toit 2017*. Tartu: Ecoprint, 112–113.

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6.3. Popular science articles / populaarteaduslikud artiklid

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VIIS VIIMAST KAITSMIST

LEA TUVIKENE

THE EFFECT OF NATURAL VARIABILITY ON THE ASSESSMENT OF ECOLOGICAL STATUS OF SHALLOW LAKES

LOODUSLIKU MUUTLIKKUSE MÕJU MADALATE JÄRVEDE ÖKOSEISUNDI HINDAMISELE

Juhtivteadur **Peeter Nõges**

16. veebruar 2018

SHUAI LI

INDUCTION OF VOLATILE ORGANIC COMPOUND EMISSIONS FROM LEAVES UPON OZONE AND METHYL JASMONATE (MEJA) TREATMENTS

TAIMELEHTEDE LENDUVÜHENDITE EMISSIOONI INDUKTSIOON OSOONI JA METÜÜL JASMONAADI MÕJUL

Professor **Ülo Niinemets**

26. veebruar 2018

KANAGENDRAN AROORAN

DIFFERENTIAL REGULATION OF RELEASE OF LEAF STRESS VOLATILES: FROM TERPENE SYNTHASE GENE EXPRESSION TO EMISSION RESPONSES UNDER HEAT, OZONE AND WOUNDING STRESSES

BIOGEENSETE LENDUVÜHENDITE EMISSIOONI REGULATSIOON STRESSITINGIMUSTES: GEENIEKSPRESSIOONIST LENDUVÜHENDITE EMISSIOONIVASTUSTENI ERINEVATE ABIOOTILISTE STRESSIDE KORRAL

Professor **Ülo Niinemets**

9. märts 2018

KAAREL SOOTS

PROCESSING TECHNOLOGY FOR POST-HARVESTED CULTIVATED BERRIES

KULTUURMARJADE KORISTUSJÄRGSE TÖÖTLEMISE TEHNOLOOGIA

Professor **Jüri Olt**

6. aprill 2018

ANDRES JÄÄRATS

THE EFFECT OF PLANTING STOCK AND SOIL SCARIFICATION ON FOREST REGENERATION

ISTUTUSMATERJALI JA MAAPINNA ETTEVALMISTAMISE MÕJU METSA UUENDAMISELE

Emeriitdotsent **Heino Seemen**, dotsent **Ivar Sibul**, **Arvo Tullus** (Tartu Ülikool)

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